# SIRTUIN-3 (SIRT3), A NOVEL POTENTIAL THERAPEUTIC TARGET FOR HEAD AND NECK CANCER

**by**

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## DEDICATION

To my beloved parents Yousef Alhazzazi and Faiza Abduljabbar. This accomplishment was made possible just because of your limitless love, sacrifice, and support during the years.

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Description of my contribution to the work presented in this dissertation:

I wrote all chapters of this dissertation. **In Chapter I**, I summarized the specific aims, and background and significance of my work.

The work of **Chapter II** has been published in Cancer (2011) Apr 15;117(8):1670-8. Drs. Kapila and Kamarajan contributed to the experimental design and manuscript editing. Dr. Kamarajan also helped with the *in vivo* experiments. Dr. Nam Joo helped with the ELISA data. Dr. Nisha D'Silva helped with the Immunohistochemical data scoring and taught us how to use the murine floor-of-mouth model to induce oral cancer tumors for our *in vivo* data. Drs. Verdin and Jing-Yi Huang provided us with SIRT3 antibodies and reagents. Sindhu Halubai and Lingling Zhang helped with the statistical analysis. Drs. Verdin, David Lombard, Jacques Nör, and Jill Macoska also helped with their useful comments throughout the publication process.

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**Chapter VI** contains summary of all the major findings of this dissertation work and future directions.

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## ABSTRACT

Head and Neck cancer is the eighth most common cancer worldwide, and squamous cell carcinoma represents the majority of the head and neck cancer cases. Despite the advancements in therapeutic approaches and treatments to treat this devastating disease, head and neck squamous cell carcinoma (HNSCC) still holds one of the worst 5-year survival rates compared to other cancer types worldwide, averaging 50%. This underscores the urgent need to explore new areas of research and develop new therapeutic drugs and approaches that can help improve the survival rate of head and neck cancer patients.

Sirtuins (SIRT1–7), class-III NAD-dependent histone deacetylases (HDAC), are involved in a myriad of cellular and tissue functions, such as regulating oxidative stress, repairing DNA, increasing genomic stability, and modulating cell survival, apoptosis, development, metabolism, and aging. Interestingly, sirtuins have emerged as critical modulators of different tumorigenic processes, thus highlighting their importance in the area of cancer research. In addition, the role of sirtuins in head and neck cancer has not yet been investigated. Therefore, because of their emerging role in cancer biology and the need to explore novel approaches to improve the fate of head and neck cancer patients, sirtuins were explored in the context of HNSCC.

In this dissertation work, we show a novel role for sirtuins, and specifically, sirtuin-3 (SIRT3), in head and neck cancer tumorigenesis. We found that of all the sirtuin family members, SIRT3 was overexpressed in oral squamous cell carcinoma (OSCC) *in vitro* and *in vivo*, compared to other sirtuins. Downregulation of SIRT3 inhibited OSCC cell growth and proliferation, and increased their sensitivity to radiation and cisplatin treatments *in vitro*.

SIRT3 downregulation reduced tumor burden *in vivo*. In addition, we found a link between SIRT3 and anoikis, apoptotic cell death triggered by loss of extracellular matrix (ECM) contacts. Our data showed that SIRT3 and receptor interacting protein (RIP) are oppositely expressed in OSCC, and that OSCC cells escaped anoikis by forming multicellular aggregates or oraspheres to maintain their survival compared to single cells, which underwent anoikis-mediated cell death. Moreover, anoikis-resistant OSCC cells that possess higher SIRT3 and lower RIP expression induced an increased tumor burden and incidence in mice, unlike their adherent OSCC cell counterparts. Stable suppression of SIRT3 also inhibited anoikis-resistance and reduced tumor incidence.

To our knowledge, there are no published reports on clinical trials using class-III HDAC inhibitors of sirtuins to treat cancer. Therefore, we investigated the effect of a novel SIRT3 inhibitor, LC-0296, on HNSCC cells that were radio-resistant and originated from patients that did not respond to conventional therapies. Interestingly, LC-0296, showed specificity toward retarding HNSCC cell survival and enhancing apoptosis, without affecting normal human oral keratinocytes. Additionally, LC-0296 not only worked as a single agent on HNSCC cells, but also synergistically when combined with either radiation or cisplatin treatments. This inhibitory effect of LC-0296 on HNSCC cells was, at least in part, mediated by modulation of ROS levels.

Lastly, because controversy has emerged in the area of sirtuin biology with regards to the role of specific sirtuins in tumorigenesis, we conducted a review of this field. Our findings highlight that several sirtuins, including SIRT1 and SIRT3, have unique roles in tumorigenesis, which appear to be cell-type and tumor-type specific. Undoubtedly, these

features need to be further explored and future studies will help shed additional knowledge in this area.

In summary, this dissertation work highlights a novel role for sirtuins, and specifically, SIRT3 in head and neck cancer tumorigenesis. Our data suggest that the development of new therapies that would specifically target SIRT3, may have a promising role in the treatment of HNSCC, and hopefully improve the survival rate of head and neck cancer patients.

# CHAPTER I

# INTRODUCTION

## Problem Statement

Head and neck squamous cell carcinoma (HNSCC) is the type of cancer that originates from the oral and nasal cavities and sinuses, lips, salivary glands, pharynges, or larynges. Squamous cell carcinoma represents the majority of the HNSCC and it is the eighth most common cancer worldwide [1]. It is also important to realize that because oral squamous cell carcinoma (OSCC) represents the majority of HNSCC cases, we find that in the literature the wording HNSCC and oral cancer is often used interchangeably, and their statistics are overlapped. Thus, in this dissertation work, the two words are used interchangeably.

Although other cancers, such as breast, prostate, and colon cancers occur more frequently than HNSCC, the 5-year survival rate of this devastating disease is poor and worse than these other cancers, averaging fifty percent [1]. In U.S., one person is estimated to die every hour from oral cancer [1], whereas in Canada, three people are expected to die from oral cancer every hour [2]. Despite advances in technology and improved therapeutic approaches to treat this disease, shortcomings remain, especially in treating aggressive and metastatic diseases and in predicting individual responses to treatment. These observations underscore the complexity of this disease and the need for personalized cancer therapy to increase the efficacy of treatment in individual cancer patients [3]. Therefore, discovering new prognostic markers and pathways that regulate cancer processes in general, and head and neck cancer specifically, is crucial to developing better approaches for cancer prevention and treatment.

The study of sirtuins (SIRT1–7) in cancer tumorigenesis and therapy is an exciting and promising new area in cancer research [4,5]. Cancer cells possess six common traits including self-sufficiency in growth signals, insensitivity to antigrowth signals, ability to evading apoptosis, promotion of sustained

angiogenesis, limitless replicative potential, and ability to invade tissue and metastasize [6]. In addition, emerging hallmarks and enabling characteristics in cancer cells include dysregulation of cellular energy and avoidance of immune distraction, and the consequences of genomic instability and tumor-promoting inflammation are factors that contribute to creating a tumorigenic microenvironment, thus further facilitating and supporting the unique features of a cancer cell phenotype [7]. The fact that sirtuins can regulate most of these cancer processes, implicates sirtuins as a novel potential therapeutic target to treat cancer [8]. However, the role of sirtuins in cancer tumorigenesis has been controversial, and specifically, the role of SIRT1 and SIRT3. Thus, further studying this new area of research will advance the field and help us to better understand the mechanisms that sirtuins use to regulate the different steps of cancer processes. Although, the literature supports an important role for sirtuins in cancer development and progression, their role in head and neck cancer has not yet been investigated. In this dissertation work, we demonstrated that sirtuins, and specifically SIRT3, is critical to head and heck cancer aggressiveness and tumorigenesis. In addition, the modulation of SIRT3 expression levels or the use of SIRT3 specific inhibitors increases the sensitivity of HNSCC to radiation and chemotherapeutic treatments, thus suggesting SIRT3 as a potential therapeutic for head and neck cancer.

## General Hypothesis

Sirtuins play a critical role in head and neck cancer aggressiveness and tumorigenesis, and sirtuins can be used as novel, sole, or adjunctive therapeutic targets to treat head and neck cancer.

## Specific Aims

Our general hypothesis will be tested by addressing the following specific aims:

**Aim 1:** To investigate the role of sirtuins in modulating head and neck cancer cell proliferation and survival, and whether the modulation of sirtuins would enhance the sensitivity of head and neck cancer to radiation and chemotherapeutic treatments. (Chapter II and Chapter IV)

**Aim 2:** To investigate whether sirtuins can modulate head and neck cancer tumorigenesis *in vivo*. (Chapter II and Chapter III)

**Aim 3:** To identify a plausible mechanism by which sirtuins inhibitors can overcome head and neck cancer tumorigenesis, thus implicating their use as a potential therapeutics in head and neck cancer. (Chapter IV and V)

## Background and Significance

### Oral Cancer; Incidence and Risk Factors

Oral cancer is a multifactorial and multistep disease that represents an abnormal growth in areas that include the tongue, floor of the mouth, lip, and throat, and with the potential to spread to other areas of the body. This abnormal cell growth is caused by an imbalance and dysregulation of pro-survival and pro-apoptotic factors. This ultimately results in the accumulation of changes, which end with the transformation of normal cells to malignant cells [9,10]. It is believed that a normal cell needs three to six newly acquired mutations to undergo this cellular transformation, and the histological progression of oral cancer carcinogenesis reflects the accumulation of these acquired changes [11,12]. These changes start with the influence of combined risk factors in the affected individual. In the U.S., approximately 35,000 people will be diagnosed with oral cancer each year and about 7,600 will die from this disease [13]. Early detection is the key to a good prognosis and survival. When oral cancer is diagnosed at an early and localized stage, the 5-year survival rate is ~82 %, however, more than half of oral cancer cases are diagnosed late after spread to near by tissues, thus dropping the 5-year survival rate to ~ 50% or even to 28% when there is metastasis to distant organs [13]. Oral cancer is twice as prevalent in men as it in women, and African American men have the highest risk of developing oral cancer compared to others in the U.S. [13].

Risk factors for oral cancer include tobacco and alcohol use, exposure to human papillomavirus (HPV), inadequate diet, prolonged sun exposure, family history of the disease, genetic alterations in

susceptible individuals, and older age, above forty. Unfortunately, however, 25% of all oral cancer patients have no obvious risk factors [14].

The incidence of this multifactorial and multistep disease also depends on the combination of different risk factors. For instance, the use of both tobacco and alcohol would significantly raise the risk compared to either one alone [15]. Thus, chewing tobacco and drinking alcohol would increase the risk of oral cancer about 24 fold in a population, such as the population in India [16]. HPV, and specifically HPV-16 contributes to 90% of all oropharyngeal cancer types, and it is responsible for the development of about 20-30% of all oral cancer cases, especially in the non-smoker or non-alcohol drinking groups [14]. Indeed, the combination of both HPV and alcohol consumption work synergistically to increase the risk of head and neck cancer patients [17].

**Oral Cancer; Biomarkers, Prognosis, and Treatment outcome**

The identification of oral cancer biomarkers is a crucial tool to enhance diagnosis, prognosis, and treatment outcome of this devastating disease. These could help identify genetic and molecular changes related to early, intermediate, and late stages of oral carcinogenesis, and could also be used to evaluate the efficacy and safety of different treatment measures, thus helping the development of personalized cancer therapy for each patient. Because of the complexity of the oral cancer process, there is no doubt that looking for a single biomarker to assess the previously mentioned parameters is not enough; however, the use of several plausible biomarkers that would control this disease in a specific patient would be a logical approach. Thus, research aimed at discovering these cancer biomarkers will help us to better understand the biology of this disease and enhance future treatment of patients with oral cancer [18]. Several methods have been used to elucidate cancer biomarkers to assess prognosis and treatment outcomes for oral cancer patients. These include the use of DNA-microarray technology to obtain gene expression profiling to help identify oral cancer specific signatures [19,20,21], immunohistochemistry and enzyme-linked immunosorbent assay (ELISA) assays to look for protein expression in OSCC tissue samples. In addition,

proteomic methods such as 2-D gel electrophoresis and mass spectrometry have been used to assess body fluid proteins in saliva and plasma [22,23]. These methods look for changes in key regulatory genes/proteins, including tumor promoters or suppressors, in oral cancer carcinogenesis, or identify epigenetic changes, including methylation or acetylation of specific proteins or tumor suppressor genes, such as p16, p53, and E-cadherin [18,24,25]. Studies in this area showed that the expression levels of p16, a gene that controls cell cycle progression in the G1 phase, is decreased in OSCC and seems to be critical for malignant progression when deleted. Decreased levels of p16 or deletion of p16 were also associated with increased recurrence rates, metastasis, and reduced survival rate [26]. The tumor suppressor p53, is frequently mutated in more than 50% of OSCC, and these mutations were associated with high expression and accumulation of p53 when assessed with immunohistochemistry. This positive expression has been reported in about 50% of adjacent non-tumor tissues, suggesting that p53 mutations are early events in the course of OSCC carcinogenesis, and they may be useful biomarkers for early detection [27]. The low expression of the adhesion molecule E-cadherin was correlated with poor prognosis and survival in OSCC. In addition, reduced levels of E-cadherin were a predictive biomarker for lymph node metastasis in oral cancer patients [28]. Epigenetics is also an emerging area of research that explores the mitotically and/or meiotically heritable changes in gene functions that cannot be explained by changes in DNA sequence. Interestingly, the two most important risk factors in oral cancer carcinogenesis, tobacco and alcohol consumption have been shown to directly modulate epigenetic factors and work synergistically to increase the risk of oral carcinogenesis [24,29]. The tumor suppressor p73, a protein related to p53 structurally and functionally, was found to be inactivated with methylation in OSCC and this silencing was associated with OSCC progression [30]. Furthermore, methylation of p16 and E-cadherin was associated with the duration of smoking and alcohol consumption, and increased tumor size and potential metastasis [29]. As previously mentioned, one strongly emerging risk factor for oropharyngeal cancer is exposure to HPV [31]. Interestingly, although the association between HPV and OSCC development is strong, some investigators consider this type of tumor of a distinct clinical and pathological nature

because these tumors, surprisingly, respond better to conventional treatments and have a better prognosis and survival rate. Therefore, HPV is considered a new prognostic biomarker for head and neck cancer [32,33]. Additionally, chromosomal aberrations such as amplifications, deletions, and structural rearrangements are another important hallmarks of oral cancer carcinogenesis [18]. These acquired events are commonly associated with loss of heterozygosity (LOH) in chromosomes 3 and 9 in the early stages of normal cell transformation. The events can be assessed with comparative genomic hybridization and microsatellite panel assessment studies. These are developing and promising areas of research aimed at discovering prognostic markers and patients at high risk for developing the disease [18,34].

The current gold standard for prediction of prognosis and for decision of treatment options is based on the TNM classification: tumor size (T), regional lymph nodal involvement (N), and the presence or absence of distant metastasis (M). Although this system is not perfect and has its limitations [35,36], it is still widely used and accepted for planning, prognosticating, and comparison of treatment outcomes. For example, tumors of similar size may behave differently due to their genomic and biological background dysregulation differences. Based on the TNM system, the tumor will then be assigned, a stage (I-IV), which will determine the treatment modality used in each specific case [18,37]. Surgical intervention is still the primary treatment option when the lesion is localized, however with advanced cases and difficult operating areas, radiation and chemotherapy is essential. Unfortunately, this type of treatment always increases the side-effects and morbidity of the disease. Nguyen and Yueh found 1-year survival rates of 60% (stage I) and 32% (stage IV) in patients with recurrence or secondary primary cancer [36]. This highlights the importance of early detection and diagnosis of the disease, and the urgent need for valid and more reliable biomarkers to better assess the nature and behavior of each specific lesion. In general, increased tumor thickness, extracapsular spread, poor histological tumor differentiation, unclear surgical margins, and angiogenesis enrichment, are all predictive factors of a poor prognosis and reduced survival rate [37,38]. In addition, Murthy *et al.* demonstrated that the location of the OSCC and the selected mode of treatment would also affect the prognosis and survival rate in advance stages of oral cancer [39]. For

example, superior outcomes were achieved in cases treated with surgery followed by post-operative radiation (PORT) compared to radiation alone. In addition, the lip and hard palate showed better outcomes and response to treatment than tongue and floor of the mouth sites. This emphasizes the importance of adapting the treatment to the tumor stage and location [39].

In summary, the current system used for OSCC tumor staging, treatment planning, and prognostication, has limitations. The urgent need for discovering valid and reliable biomarkers to control this disease and assess the previously mentioned parameters is ever present. These biomarkers may help better evaluate preventive and therapeutic measures, aid in the early diagnosis of the disease, and reduce the number of patients that would undergo either unnecessary or deficient treatment regimens, and hopefully help advance the field toward the development of personalized cancer therapy. These biomarkers can then either replace or add to the current TNM system and become part of routinely used approaches for diagnosis, prognosis and treatment planning.

**Sirtuins and Cancer; an Overview**

Sirtuins (SIRT1–7) are the mammalian homologues of the silent information regulator 2 (Sir2) first discovered in *Saccharomyces cerevisiae* as an $NAD^+$-dependent histone deacetylase (HDAC). They are classified as class III HDACs: they require $NAD^+$ as a cofactor to exert their biological function. They contain an evolutionarily conserved core domain, which is essential for their activity as NAD-dependent deacetylases or ADP-ribosyltransferases [40,41]. Sirtuin biology is complex, and sirtuins are widely expressed in normal tissues [42]. They are involved in a myriad of cellular and tissue functions, such as regulating oxidative stress, repairing DNA, increasing genomic stability, and modulating cell survival, apoptosis, development, metabolism, aging and longevity [4,5]. Some sirtuins are located in different cellular compartments (Fig. 1.1). Those in the same compartment, such as the mitochondrial SIRT3, 4, and 5, have different sequences, and thus, unique and diverse cellular functions, and can interact with different targets [5,40,41].

SIRT1 is the best-characterized member of the mammalian sirtuins. It is located predominately in the nucleus and modulates cellular stress and survival by deacetylating p53 [43,44], FOXO, and Ku70 [45,46], thus promoting tumorigenesis. SIRT1 is thought to have a role in skin, colon, breast and lung cancer, via one or more of these mentioned targets [47,48,49,50,51]. It also regulates vascular endothelial homeostasis, thereby controlling angiogenesis and vascular function, [52]. Thus, it is likely crucial in regulating cell survival, and its functions may contribute to cancer tumorigenesis.

On the other hand, other studies support a tumor suppressor role for SIRT1 [53,54,55]. For example, SIRT1 mutant mice possess an impaired DNA repair response, genomic instability, and increased incidence of tumorigenesis. Moreover, SIRT1 levels were lower in breast cancer and hepatic cell carcinoma than in normal controls [54]. These studies highlight the discrepancy in the literature about the biological functions of SIRT1 and underscore the complexity of sirtuin biology (See reviews by Deng *et al.* and Lim *et al.* [56,57]).

SIRT2 is found in the cytosol, where it colocalizes with microtubules and deacetylases α-tubulin [58]. It controls cell-cycle progression [59] and is downregulated in human gliomas, suggesting a tumor suppressor role in brain cancer [60].

The gene for the nuclear protein SIRT6 is located on chromosome 19p13.3; a region frequently affected by chromosomal alterations in acute leukemia [61]. In addition, SIRT6-deficient mice possess an aging-like phenotype and genomic instability [62,63].

SIRT7, which is localized in the nucleolus and functions as a positive regulator of RNA polymerase I-mediated transcription, is required for cell proliferation and survival [64]. SIRT7 gene is located on chromosome 17q25.3; a region frequently associated with chromosomal alterations in leukemias and lymphomas [65]. SIRT7 is also upregulated in breast and thyroid cancers [66,67,68].

The remaining three sirtuins, SIRT3, SIRT4, and SIRT5, are mitochondrial sirtuins [42,69]. Although SIRT4 lacks deacetylation activity, it has weak ADP-ribosyltransferase activity [70,71] and plays an important role in insulin regulation [72]. SIRT4 knockout mice are viable, fertile, and display no

phenotype abnormalities, compared to wild-type littermates, but show increased levels of insulin secretion [70]. In contrast to SIRT1 and SIRT3, SIRT4 activity is downregulated by calorie restriction (CR) [70]. SIRT5 has less deacetylase activity than SIRT1-3 [73] and remains the least-characterized sirtuin. SIRT5 is located on chromosome 6p23, an area linked to numerous abnormalities associated with malignant diseases, such as acute myeloid leukemia [74]. In contrast to SIRT4- and SIRT5-deficient mice, SIRT3-deficient mice show greater mitochondrial hyperacetylation than wild-type mice, suggesting that SIRT3 is a key mitochondrial deacetylase [75].

The controversy over whether SIRT1 works as tumor promoter or suppressor, can also be applied to SIRT3. SIRT3 exerts a prosurvival role in multiple cancer pathways. The tumor suppressor, p53 was recently identified as a new target for SIRT3 deacetylation in bladder cancer [76]. SIRT3 rescued p53-induced growth arrest in human bladder tumor–derived EJ-p53 cells, supporting a prosurvival role for SIRT3 [76]. Ashraf *et al.* reported that increased transcriptional levels of SIRT3 were associated with lymph node–positive breast cancer, and SIRT3 expression was significantly higher in these samples than normal breast biopsies [66]. Additionally, SIRT3 is overexpressed in metabolically active tissues, such as the heart, where SIRT3 protects against genomic and stress-mediated apoptosis, at least in part, via ROS reduction and increases in Ku70-Bax interactions [77,78] These can be mechanisms by which cancer cells that overexpress SIRT3 can similarly resist cell death. This was indeed demonstrated by the same group in the HeLa cervical cancer cell line [77]. In contrast, other reports support a proapoptotic role for SIRT3. SIRT3 induces growth arrest and apoptosis in several colorectal carcinoma and osteosarcoma cells and in non-cancer human cell lines, such as retinal epithelial and lung fibroblast cells [79]. This action is mediated, in part, by SIRT3 modulation of the JNK2 signaling pathway in these cell lines [79]. In leukemia cell lines, treatment with Kaempferol, a flavonoid that auto-oxidizes and generates ROS, induces apoptosis via increasing Bax and SIRT3 levels and activating caspase-3 cascades [80]. Moreover, SIRT3 demonstrates a tumor suppressor function in breast, colon carcinoma and osteosarcoma cells, via suppressing ROS, HIF1-α and its targeted genes [81,82,83].

It seems that SIRT1 and SIRT3 may play a dual role. Their functions may vary in different normal and tumor tissues and may be cell- and tumor-type specific. Therefore, their role in specific cell types must not be generalized, but should be examined separately under different cellular events, and specifically in each cancer type to determine whether it functions as a tumor promoter or suppressor [49].

**Sirtuins as Potential Therapeutic Targets for Cancer**

Several studies have implicated sirtuins as novel therapeutic targets for many age-related diseases, including cancer, but how sirtuins are involved in cancer is still not clear and controversial. A better understanding of how individual sirtuins are involved in different cancer types is important for assessing their potential in possible therapies. Sirtuins seem to be involved in tumorigenesis, and thus, sirtuin inhibitors/modifiers might have therapeutic benefit. Several inhibitors and activators of sirtuins have been tested in different cancer cell lines, but few have been tested *in vivo* [84]. The sirtuin inhibitors, sirtinol and splitomicin, induced senescence-like growth arrest in breast and lung cancers [85]. NAM, another sirtuin inhibitor, induced apoptosis in lung cancer [43]. Treatment of B-cell lymphoma cells with cambinol, a SIRT1 inhibitor, inhibited tumor cell growth and induced apoptosis *in vitro,* and reduced tumor size compared to controls *in vivo* [86]. Moreover, cambinol sensitized lung cancer cells to the DNA-damaging agent etoposide, thus inducing cell death and etoposide-induced cell-cycle arrest [86] (See review by Balcerczyk *et al*. [87]).

Resveratrol, a polyphenol phytoalexin and natural component found in the skin of red grapes and red wine, works as an activator of sirtuins and possesses diverse natural therapeutic benefits, including cardiac protection, anti-inflammatory and anti-carcinogenic effects, preventing obesity, and promoting longevity [88,89]. These therapeutic benefits seem to work, at least in part, by activating SIRT1 and SIRT3, although it is not yet clear whether these effects are mediated by direct or indirect mechanisms [88,89,90,91]. Interestingly, resveratrol modulates both survival and death signals, depending on the administered dose *in vivo* [92]. At low doses (2.5 or 5 mg/kg for 14 days in rats), resveratrol provided

cardiac protection and lower levels of apoptosis than controls. In contrast, at high doses (25 or 50 mg/kg), resveratrol hindered cardiac function and promoted apoptosis of cardiomyocytes. The former effect was mediated by augmenting survival signaling pathways, including p-Akt, NFκB, and Bcl-2 activation. The later was mediated by switching on the death program by repressing the same pathways [92]. Moreover, resveratrol is the most studied sirtuin activator in cancer prevention. Many studies have shown resveratrol to be a natural anticarcinogenic agent, modulating different stages of cancer, including initiation, promotion, and progression in neuroblastoma, hepatoma, breast, lung, pancreatic, and prostate cancers *in vitro* or *in vivo* [93,94]. However, many of these studies yielded contradictory results even in the same tumor type. The reasons for these discrepancies could be due to the different experimental approaches used to examine resveratrol. For instance, some data were collected from mice and others from rats. Some studies used animal carcinogenesis models with different genetic backgrounds, others used different doses of resveratrol, and yet others used different time frames for drug administration. Resolving these discrepancies would be very helpful. In addition, these drugs are all generalized inhibitors or activators of several sirtuin family members. Thus, some redundancy or even opposing actions of some sirtuin functions may be expected. Furthermore, different tumors have different genetic backgrounds that differ from one person to another. This diversity may explain why one patient responds well to a specific treatment but another patient with the same type of cancer does not.

Therefore, the rapidly evolving era of personalized and targeted approaches holds great promise for the future of cancer therapy. Foe example, looking for specific cancer profile signatures by using gene microarray technology as an example [21], and then identifying key dysregulated pathways for targeted therapy, would be beneficial and result in less oral complications than using conventional therapeutic approaches alone [95,96]. Some of these targeted therapies currently used in phase I-III to trials to treat oral cancer patients, includes the use of anti-tumor monoclonal antibodies, small molecules, and signal transduction receptor inhibitors [95]. For example these include the epidermal growth factor receptor inhibitor (EGFR), erlotinib, and the anti-vascular endothelial growth factor (VEGF) antibody,

bevcizumab (Avastin). The former is a small molecule tyrosine kinase inhibitor (TKIs) of EGFR, and the latter is an antibody that inhibits angiogenesis, thus preventing tumor growth by neutralizing VEGF activity. Both are used in combination to treat patients with recurrent and metastatic OSCC. This combination is well tolerated and shows promising results [97]. In addition, the use of RNA interference (RNAi) as a specifically targeted therapeutic approach may also be useful especially in combination with conventional treatments. Currently, studies using RNAi are still in early stages of clinical trials. RNAi has been tested in different types of cancer, such as lung, advanced liver, and chronic myeloid leukemia. This demonstrates the usefulness of targeted gene knockdown as a potential therapeutic approach (See review by Phalon *et al.* [98]).

Class-I and II HDAC inhibitors have been tested in phase-I and II clinical trials with or without conventional chemotherapeutic drugs. The agents were well tolerated with low toxicity and yielded promising results. Some of the agents used include phenyl acetate, suberoylanilide hydroxamic acid (SAHA), and Trichostatin (TSA) to treat patients with solid tumors, hematologic malignancies, and advanced leukemias [99]. In head and neck cancer, SAHA and TSA show selective inhibition of cell growth and induction of apoptosis compared to normal and premalignant cells [100,101]. In addition, the combination of SAHA and cisplatin chemotherapeutic drugs possess synergetic cytotoxicity effects against OSCC cells, at least in part, by enhancing ROS production [102].

To our knowledge, there are no published reports on clinical trials using class-III HDAC inhibitors of sirtuins to treat cancer. However, class-III HDAC activators of sirtuins such as resveratrol are currently in early stages of clinical trials, and have been tested for safety and potential treatment of age-related diseases such diabetes, neurodegenerative disorders and cancer (See references [103,104] for ongoing and published clinical trials).

In summary, head and neck cancer still holds one of the worse survival rates compared to other cancer types worldwide. This underscores the need for discovering new areas of research that can help improve the survival rate of head and neck cancer patients. The role of sirtuins (SIRT1-7) in cancer has

emerged to be an interesting and important area of cancer research, however, because of the controversy in this area, there is a lot yet to be revealed. In addition, the role of sirtuins in head and neck cancer has not yet been investigated, therefore, it will be interesting to determine if this field of study could hold a new hope for patients suffering from this devastating disease.

## Organization of Thesis Contents

In **Chapter II,** the initial evidence of the potential role of sirtuins (SIRT1-7) in oral cancer tumorigenesis is discussed. Specifically, the evidence showing that SIRT3 is a key regulator of OSCC cell survival and proliferation, and that SIRT3 inhibition enhances the sensitivity of oral cancer cells to radiation and chemotherapeutic treatments is demonstrated. In addition, evidence that SIRT3 controls the aggressiveness of oral cancer tumorigenesis *in vivo* is also shown.

In **Chapter III**, it was demonstrated previously by our group that receptor interacting protein (RIP) has a role in controlling anoikis in oral cancer. In this work, a potential crosstalk between RIP and SIRT3 in controlling anoikis resistance and tumorigenesis is demonstrated *in vitro* and *in vivo*.

In **Chapter IV**, the role of SIRT3 in controlling HNSCC cell survival, proliferation, and apoptosis, is further demonstrated, by testing the effect of a novel SIRT3 inhibitor against HNSCC cells, and the mechanism by which this SIRT3 inhibitor may, at least in part, affect HNSCC cell survival, proliferation, and apoptosis, will be discussed.

In **Chapter V**, because the role of SIRT3 in cancer is controversial, the role of SIRT3 as a tumor promoter or suppressor in cancer is discussed.

In **Chapter VI,** summary of major findings and future directions of this dissertation work is presented.

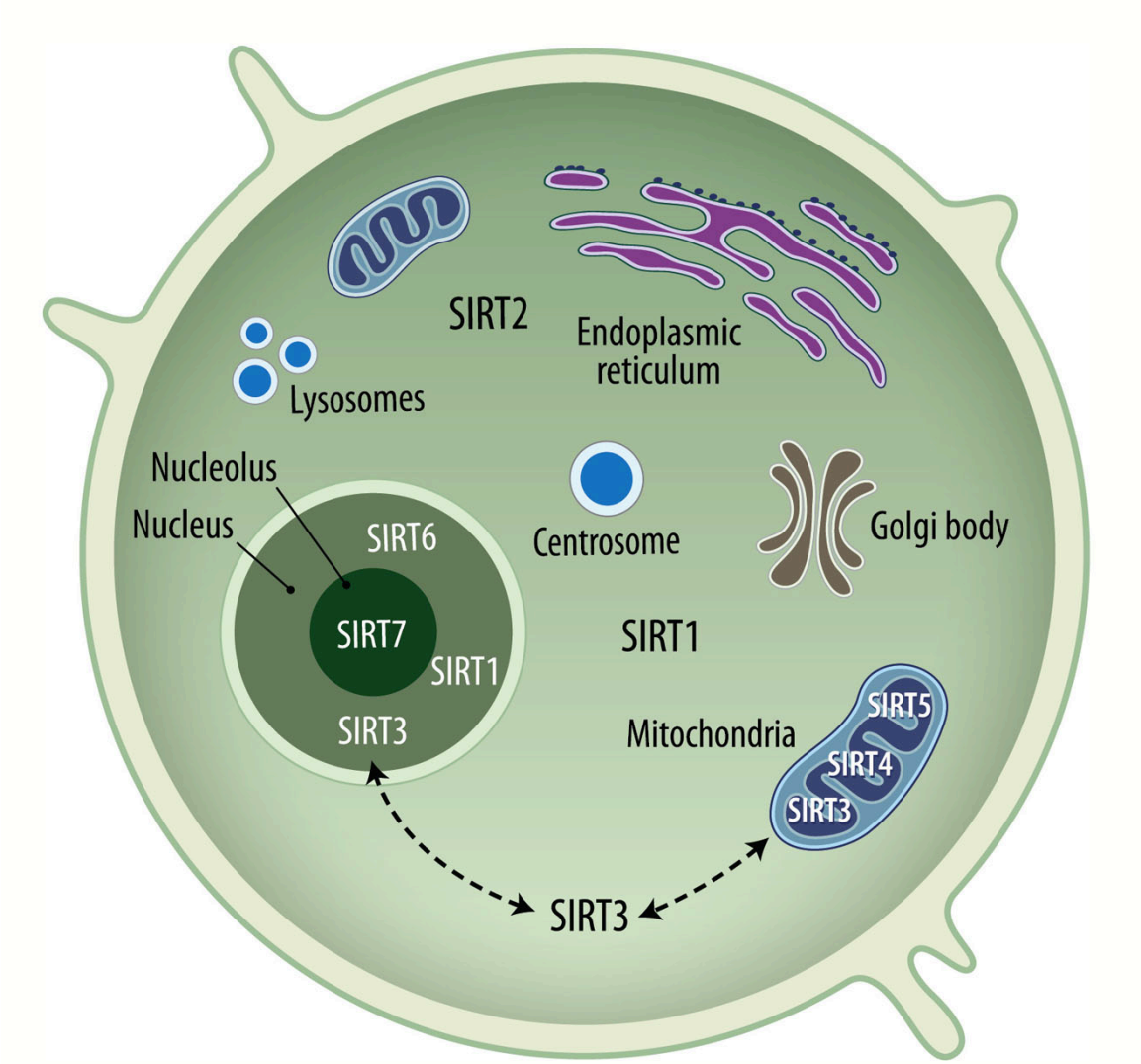


**Figure 1.1: Sirtuins subcellular localization:** SIRT1 is predominantly located in the nucleus, and also in the cytosol. SIRT2 is localized in the cytosol. SIRT3, SIRT4, and SIRT5 are mitochondrial proteins, but SIRT3 may also be found in the nucleus and cytosol under different cellular events. SIRT6 and SIRT7 are localized in the nucleus and nucleolus, respectively.

**References**


[1] http://www.cancer.org/, (Accessed 8-20-11).
[2] http://www.cancer.ca/, (Accessed 8-22-11).
[3] P. Workman, J. de Bono, Targeted therapeutics for cancer treatment: major progress towards personalised molecular medicine, Curr Opin Pharmacol 8 (2008) 359-362.
[4] L.R. Saunders, E. Verdin, Sirtuins: critical regulators at the crossroads between cancer and aging, Oncogene 26 (2007) 5489-5504.
[5] S. Michan, D. Sinclair, Sirtuins in mammals: insights into their biological function, Biochem J 404 (2007) 1-13.
[6] D. Hanahan, R.A. Weinberg, The hallmarks of cancer, Cell 100 (2000) 57-70.
[7] D. Hanahan, R.A. Weinberg, Hallmarks of cancer: the next generation, Cell 144 (2011) 646-674.
[8] D. McGuinness, D.H. McGuinness, J.A. McCaul, P.G. Shiels, Sirtuins, bioageing, and cancer, Journal of aging research 2011 (2011) 235754.
[9] S.I. Pai, W.H. Westra, Molecular pathology of head and neck cancer: implications for diagnosis, prognosis, and treatment, Annual review of pathology 4 (2009) 49-70.
[10] D.T. Wong, R. Todd, T. Tsuji, R.B. Donoff, Molecular biology of human oral cancer, Crit Rev Oral Biol Med 7 (1996) 319-328.
[11] B. Vogelstein, K.W. Kinzler, The multistep nature of cancer, Trends in genetics : TIG 9 (1993) 138-141.
[12] E.E. Vokes, R.R. Weichselbaum, S.M. Lippman, W.K. Hong, Head and neck cancer, The New England journal of medicine 328 (1993) 184-194.
[13] http://www.nidcr.nih.gov/NR/rdonlyres/3DBC0B3D-6425-49AB-8B20-74029DF1B515/0/OralCancerStatistics.pdf, (Accessed 8-20-11).
[14] http://www.cancer.org/acs/groups/content/@nho/documents/document/oralcancerpdf.pdf, (Accessed 8-20-11).
[15] A.G. Zygogianni, G. Kyrgias, P. Karakitsos, A. Psyrri, J. Kouvaris, N. Kelekis, V. Kouloulias, Oral squamous cell cancer: early detection and the role of alcohol and smoking, Head & neck oncology 3 (2011) 2.
[16] A. Znaor, P. Brennan, V. Gajalakshmi, A. Mathew, V. Shanta, C. Varghese, P. Boffetta, Independent and combined effects of tobacco smoking, chewing and alcohol drinking on the risk of oral, pharyngeal and esophageal cancers in Indian men, International journal of cancer. Journal international du cancer 105 (2003) 681-686.
[17] E.M. Smith, J.M. Ritchie, K.F. Summersgill, H.T. Hoffman, D.H. Wang, T.H. Haugen, L.P. Turek, Human papillomavirus in oral exfoliated cells and risk of head and neck cancer, Journal of the National Cancer Institute 96 (2004) 449-455.
[18] S.D. Silva, A. Ferlito, R.P. Takes, R.H. Brakenhoff, M.D. Valentin, J.A. Woolgar, C.R. Bradford, J.P. Rodrigo, A. Rinaldo, M.P. Hier, L.P. Kowalski, Advances and applications of oral cancer basic research, Oral oncology (2011).
[19] E. Mendez, C. Cheng, D.G. Farwell, S. Ricks, S.N. Agoff, N.D. Futran, E.A. Weymuller, Jr., N.C. Maronian, L.P. Zhao, C. Chen, Transcriptional expression profiles of oral squamous cell carcinomas, Cancer 95 (2002) 1482-1494.
[20] C.E. Schmalbach, D.B. Chepeha, T.J. Giordano, M.A. Rubin, T.N. Teknos, C.R. Bradford, G.T. Wolf, R. Kuick, D.E. Misek, D.K. Trask, S. Hanash, Molecular profiling and the identification of genes associated with metastatic oral cavity/pharynx squamous cell carcinoma, Archives of otolaryngology--head & neck surgery 130 (2004) 295-302.

[21] X. Liu, A. Kolokythas, J. Wang, H. Huang, X. Zhou, Gene Expression Signatures of Lymph Node Metastasis in Oral Cancer: Molecular Characteristics and Clinical Significances, Current cancer therapy reviews 6 (2010) 294-307.
[22] R. Vitorino, M.J. Lobo, A.J. Ferrer-Correira, J.R. Dubin, K.B. Tomer, P.M. Domingues, F.M. Amado, Identification of human whole saliva protein components using proteomics, Proteomics 4 (2004) 1109-1115.
[23] S. Hu, Y. Xie, P. Ramachandran, R.R. Ogorzalek Loo, Y. Li, J.A. Loo, D.T. Wong, Large-scale identification of proteins in human salivary proteome by liquid chromatography/mass spectrometry and two-dimensional gel electrophoresis-mass spectrometry, Proteomics 5 (2005) 1714-1728.
[24] R. Radhakrishnan, S. Kabekkodu, K. Satyamoorthy, DNA hypermethylation as an epigenetic mark for oral cancer diagnosis, Journal of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology (2011).
[25] T. Sakuma, K. Uzawa, T. Onda, M. Shiiba, H. Yokoe, T. Shibahara, H. Tanzawa, Aberrant expression of histone deacetylase 6 in oral squamous cell carcinoma, International journal of oncology 29 (2006) 117-124.
[26] A.K. El-Naggar, S. Lai, G.L. Clayman, J.H. Zhou, S.A. Tucker, J. Myers, M.A. Luna, W.F. Benedict, Expression of p16, Rb, and cyclin D1 gene products in oral and laryngeal squamous carcinoma: biological and clinical implications, Human pathology 30 (1999) 1013-1018.
[27] J.C. Ahomadegbe, M. Barrois, S. Fogel, M.L. Le Bihan, S. Douc-Rasy, P. Duvillard, J.P. Armand, G. Riou, High incidence of p53 alterations (mutation, deletion, overexpression) in head and neck primary tumors and metastases; absence of correlation with clinical outcome. Frequent protein overexpression in normal epithelium and in early non-invasive lesions, Oncogene 10 (1995) 1217-1227.
[28] A. Bankfalvi, M. Krassort, A. Vegh, E. Felszeghy, J. Piffko, Deranged expression of the E-cadherin/beta-catenin complex and the epidermal growth factor receptor in the clinical evolution and progression of oral squamous cell carcinomas, Journal of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology 31 (2002) 450-457.
[29] M. Hasegawa, H.H. Nelson, E. Peters, E. Ringstrom, M. Posner, K.T. Kelsey, Patterns of gene promoter methylation in squamous cell cancer of the head and neck, Oncogene 21 (2002) 4231-4236.
[30] D. Araki, K. Uzawa, T. Watanabe, M. Shiiba, A. Miyakawa, H. Yokoe, H. Tanzawa, Frequent allelic losses on the short arm of chromosome 1 and decreased expression of the p73 gene at 1p36.3 in squamous cell carcinoma of the oral cavity, International journal of oncology 20 (2002) 355-360.
[31] G. Pannone, A. Santoro, S. Papagerakis, L. Lo Muzio, G. De Rosa, P. Bufo, The role of human papillomavirus in the pathogenesis of head & neck squamous cell carcinoma: an overview, Infectious agents and cancer 6 (2011) 4.
[32] S.F. Chen, F.S. Yu, Y.C. Chang, E. Fu, S. Nieh, Y.S. Lin, Role of human papillomavirus infection in carcinogenesis of oral squamous cell carcinoma with evidences of prognostic association, Journal of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology (2011).
[33] A.R. Kreimer, G.M. Clifford, P.J. Snijders, X. Castellsague, C.J. Meijer, M. Pawlita, R. Viscidi, R. Herrero, S. Franceschi, HPV16 semiquantitative viral load and serologic biomarkers in oral and

oropharyngeal squamous cell carcinomas, International journal of cancer. Journal international du cancer 115 (2005) 329-332.
[34] H.T. Chien, C.T. Liao, S.F. Huang, I.H. Chen, T.Y. Liu, Y.S. Jou, H.M. Wang, L.L. Hsieh, Clinical significance of genome-wide minimally deleted regions in oral squamous cell carcinomas, Genes, chromosomes & cancer 50 (2011) 358-369.
[35] D. Marsh, K. Suchak, K.A. Moutasim, S. Vallath, C. Hopper, W. Jerjes, T. Upile, N. Kalavrezos, S.M. Violette, P.H. Weinreb, K.A. Chester, J.S. Chana, J.F. Marshall, I.R. Hart, A.K. Hackshaw, K. Piper, G.J. Thomas, Stromal features are predictive of disease mortality in oral cancer patients, The Journal of pathology 223 (2011) 470-481.
[36] T.V. Nguyen, B. Yueh, Weight loss predicts mortality after recurrent oral cavity and oropharyngeal carcinomas, Cancer 95 (2002) 553-562.
[37] J. Massano, F.S. Regateiro, G. Januario, A. Ferreira, Oral squamous cell carcinoma: review of prognostic and predictive factors, Oral Surg Oral Med Oral Pathol Oral Radiol Endod 102 (2006) 67-76.
[38] J.C. Jan, W.H. Hsu, S.A. Liu, Y.K. Wong, C.K. Poon, R.S. Jiang, J.S. Jan, I.F. Chen, Prognostic factors in patients with buccal squamous cell carcinoma: 10-year experience, Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons 69 (2011) 396-404.
[39] V. Murthy, J.P. Agarwal, S.G. Laskar, T. Gupta, A. Budrukkar, P. Pai, P. Chaturvedi, D. Chaukar, A. D'Cruz, Analysis of prognostic factors in 1180 patients with oral cavity primary cancer treated with definitive or adjuvant radiotherapy, Journal of cancer research and therapeutics 6 (2010) 282-289.
[40] R.A. Frye, Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins, Biochem Biophys Res Commun 273 (2000) 793-798.
[41] M.C. Haigis, L.P. Guarente, Mammalian sirtuins--emerging roles in physiology, aging, and calorie restriction, Genes Dev 20 (2006) 2913-2921.
[42] E. Michishita, J.Y. Park, J.M. Burneskis, J.C. Barrett, I. Horikawa, Evolutionarily conserved and nonconserved cellular localizations and functions of human SIRT proteins, Mol Biol Cell 16 (2005) 4623-4635.
[43] J. Luo, A.Y. Nikolaev, S. Imai, D. Chen, F. Su, A. Shiloh, L. Guarente, W. Gu, Negative control of p53 by Sir2alpha promotes cell survival under stress, Cell 107 (2001) 137-148.
[44] H. Vaziri, S.K. Dessain, E. Ng Eaton, S.I. Imai, R.A. Frye, T.K. Pandita, L. Guarente, R.A. Weinberg, hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase, Cell 107 (2001) 149-159.
[45] A. Brunet, L.B. Sweeney, J.F. Sturgill, K.F. Chua, P.L. Greer, Y. Lin, H. Tran, S.E. Ross, R. Mostoslavsky, H.Y. Cohen, L.S. Hu, H.L. Cheng, M.P. Jedrychowski, S.P. Gygi, D.A. Sinclair, F.W. Alt, M.E. Greenberg, Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase, Science 303 (2004) 2011-2015.
[46] H.Y. Cohen, C. Miller, K.J. Bitterman, N.R. Wall, B. Hekking, B. Kessler, K.T. Howitz, M. Gorospe, R. de Cabo, D.A. Sinclair, Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase, Science 305 (2004) 390-392.
[47] D.M. Huffman, W.E. Grizzle, M.M. Bamman, J.S. Kim, I.A. Eltoum, A. Elgavish, T.R. Nagy, SIRT1 is significantly elevated in mouse and human prostate cancer, Cancer Res 67 (2007) 6612-6618.
[48] H. Ota, M. Akishita, M. Eto, K. Iijima, M. Kaneki, Y. Ouchi, Sirt1 modulates premature senescence-like phenotype in human endothelial cells, J Mol Cell Cardiol 43 (2007) 571-579.
[49] W. Stunkel, B.K. Peh, Y.C. Tan, V.M. Nayagam, X. Wang, M. Salto-Tellez, B. Ni, M. Entzeroth, J. Wood, Function of the SIRT1 protein deacetylase in cancer, Biotechnol J 2 (2007) 1360-1368.

[50] Y. Sun, D. Sun, F. Li, L. Tian, C. Li, L. Li, R. Lin, S. Wang, Downregulation of Sirt1 by antisense oligonucleotides induces apoptosis and enhances radiation sensitization in A549 lung cancer cells, Lung Cancer 58 (2007) 21-29.
[51] Y. Hida, Y. Kubo, K. Murao, S. Arase, Strong expression of a longevity-related protein, SIRT1, in Bowen's disease, Arch Dermatol Res 299 (2007) 103-106.
[52] M. Potente, S. Dimmeler, Emerging roles of SIRT1 in vascular endothelial homeostasis, Cell Cycle 7 (2008) 2117-2122.
[53] F. Gao, J. Cheng, T. Shi, E.T. Yeh, Neddylation of a breast cancer-associated protein recruits a class III histone deacetylase that represses NFkappaB-dependent transcription, Nat Cell Biol 8 (2006) 1171-1177.
[54] R.H. Wang, K. Sengupta, C. Li, H.S. Kim, L. Cao, C. Xiao, S. Kim, X. Xu, Y. Zheng, B. Chilton, R. Jia, Z.M. Zheng, E. Appella, X.W. Wang, T. Ried, C.X. Deng, Impaired DNA damage response, genome instability, and tumorigenesis in SIRT1 mutant mice, Cancer Cell 14 (2008) 312-323.
[55] M. Fu, M. Liu, A.A. Sauve, X. Jiao, X. Zhang, X. Wu, M.J. Powell, T. Yang, W. Gu, M.L. Avantaggiati, N. Pattabiraman, T.G. Pestell, F. Wang, A.A. Quong, C. Wang, R.G. Pestell, Hormonal control of androgen receptor function through SIRT1, Mol Cell Biol 26 (2006) 8122-8135.
[56] C.X. Deng, SIRT1, is it a tumor promoter or tumor suppressor?, Int J Biol Sci 5 (2009) 147-152.
[57] C.S. Lim, SIRT1: tumor promoter or tumor suppressor?, Med Hypotheses 67 (2006) 341-344.
[58] B.J. North, B.L. Marshall, M.T. Borra, J.M. Denu, E. Verdin, The human Sir2 ortholog, SIRT2, is an NAD+-dependent tubulin deacetylase, Mol Cell 11 (2003) 437-444.
[59] S.C. Dryden, F.A. Nahhas, J.E. Nowak, A.S. Goustin, M.A. Tainsky, Role for human SIRT2 NAD-dependent deacetylase activity in control of mitotic exit in the cell cycle, Mol Cell Biol 23 (2003) 3173-3185.
[60] M. Hiratsuka, T. Inoue, T. Toda, N. Kimura, Y. Shirayoshi, H. Kamitani, T. Watanabe, E. Ohama, C.G. Tahimic, A. Kurimasa, M. Oshimura, Proteomics-based identification of differentially expressed genes in human gliomas: down-regulation of SIRT2 gene, Biochem Biophys Res Commun 309 (2003) 558-566.
[61] U. Mahlknecht, A.D. Ho, S. Voelter-Mahlknecht, Chromosomal organization and fluorescence in situ hybridization of the human Sirtuin 6 gene, Int J Oncol 28 (2006) 447-456.
[62] R. Mostoslavsky, K.F. Chua, D.B. Lombard, W.W. Pang, M.R. Fischer, L. Gellon, P. Liu, G. Mostoslavsky, S. Franco, M.M. Murphy, K.D. Mills, P. Patel, J.T. Hsu, A.L. Hong, E. Ford, H.L. Cheng, C. Kennedy, N. Nunez, R. Bronson, D. Frendewey, W. Auerbach, D. Valenzuela, M. Karow, M.O. Hottiger, S. Hursting, J.C. Barrett, L. Guarente, R. Mulligan, B. Demple, G.D. Yancopoulos, F.W. Alt, Genomic instability and aging-like phenotype in the absence of mammalian SIRT6, Cell 124 (2006) 315-329.
[63] D.B. Lombard, B. Schwer, F.W. Alt, R. Mostoslavsky, SIRT6 in DNA repair, metabolism and ageing, J Intern Med 263 (2008) 128-141.
[64] E. Ford, R. Voit, G. Liszt, C. Magin, I. Grummt, L. Guarente, Mammalian Sir2 homolog SIRT7 is an activator of RNA polymerase I transcription, Genes Dev 20 (2006) 1075-1080.
[65] S. Voelter-Mahlknecht, S. Letzel, U. Mahlknecht, Fluorescence in situ hybridization and chromosomal organization of the human Sirtuin 7 gene, Int J Oncol 28 (2006) 899-908.
[66] N. Ashraf, S. Zino, A. Macintyre, D. Kingsmore, A.P. Payne, W.D. George, P.G. Shiels, Altered sirtuin expression is associated with node-positive breast cancer, Br J Cancer 95 (2006) 1056-1061.
[67] R. Frye, "SIRT8" expressed in thyroid cancer is actually SIRT7, Br J Cancer 87 (2002) 1479.

[68] F. de Nigris, J. Cerutti, C. Morelli, D. Califano, L. Chiariotti, G. Viglietto, G. Santelli, A. Fusco, Isolation of a SIR-like gene, SIR-T8, that is overexpressed in thyroid carcinoma cell lines and tissues, Br J Cancer 86 (2002) 917-923.
[69] J.Y. Huang, M.D. Hirschey, T. Shimazu, L. Ho, E. Verdin, Mitochondrial sirtuins, Biochim Biophys Acta 1804 (2010) 1645-1651.
[70] M.C. Haigis, R. Mostoslavsky, K.M. Haigis, K. Fahie, D.C. Christodoulou, A.J. Murphy, D.M. Valenzuela, G.D. Yancopoulos, M. Karow, G. Blander, C. Wolberger, T.A. Prolla, R. Weindruch, F.W. Alt, L. Guarente, SIRT4 inhibits glutamate dehydrogenase and opposes the effects of calorie restriction in pancreatic beta cells, Cell 126 (2006) 941-954.
[71] N. Ahuja, B. Schwer, S. Carobbio, D. Waltregny, B.J. North, V. Castronovo, P. Maechler, E. Verdin, Regulation of insulin secretion by SIRT4, a mitochondrial ADP-ribosyltransferase, J Biol Chem 282 (2007) 33583-33592.
[72] C. Argmann, J. Auwerx, Insulin secretion: SIRT4 gets in on the act, Cell 126 (2006) 837-839.
[73] E. Verdin, F. Dequiedt, W. Fischle, R. Frye, B. Marshall, B. North, Measurement of mammalian histone deacetylase activity, Methods Enzymol 377 (2004) 180-196.
[74] U. Mahlknecht, A.D. Ho, S. Letzel, S. Voelter-Mahlknecht, Assignment of the NAD-dependent deacetylase sirtuin 5 gene (SIRT5) to human chromosome band 6p23 by in situ hybridization, Cytogenet Genome Res 112 (2006) 208-212.
[75] D.B. Lombard, F.W. Alt, H.L. Cheng, J. Bunkenborg, R.S. Streeper, R. Mostoslavsky, J. Kim, G. Yancopoulos, D. Valenzuela, A. Murphy, Y. Yang, Y. Chen, M.D. Hirschey, R.T. Bronson, M. Haigis, L.P. Guarente, R.V. Farese, Jr., S. Weissman, E. Verdin, B. Schwer, Mammalian Sir2 homolog SIRT3 regulates global mitochondrial lysine acetylation, Mol Cell Biol 27 (2007) 8807-8814.
[76] S. Li, M. Banck, S. Mujtaba, M.M. Zhou, M.M. Sugrue, M.J. Walsh, p53-Induced growth arrest is regulated by the mitochondrial SirT3 deacetylase, PLoS ONE 5 (2010) e10486.
[77] N.R. Sundaresan, S.A. Samant, V.B. Pillai, S.B. Rajamohan, M.P. Gupta, SIRT3 is a stress responsive deacetylase in cardiomyocytes that protects cells from stress-mediated cell death by deacetylation of Ku-70, Mol Cell Biol 28 (2008) 6384-6401.
[78] N.R. Sundaresan, M. Gupta, G. Kim, S.B. Rajamohan, A. Isbatan, M.P. Gupta, Sirt3 blocks the cardiac hypertrophic response by augmenting Foxo3a-dependent antioxidant defense mechanisms in mice, J Clin Invest 119 (2009) 2758-2771.
[79] S.J. Allison, J. Milner, SIRT3 is pro-apoptotic and participates in distinct basal apoptotic pathways, Cell Cycle 6 (2007) 2669-2677.
[80] G. Marfe, M. Tafani, M. Indelicato, P. Sinibaldi-Salimei, V. Reali, B. Pucci, M. Fini, M.A. Russo, Kaempferol induces apoptosis in two different cell lines via Akt inactivation, Bax and SIRT3 activation, and mitochondrial dysfunction, J Cell Biochem 106 (2009) 643-650.
[81] H.S. Kim, K. Patel, K. Muldoon-Jacobs, K.S. Bisht, N. Aykin-Burns, J.D. Pennington, R. van der Meer, P. Nguyen, J. Savage, K.M. Owens, A. Vassilopoulos, O. Ozden, S.H. Park, K.K. Singh, S.A. Abdulkadir, D.R. Spitz, C.X. Deng, D. Gius, SIRT3 Is a Mitochondria-Localized Tumor Suppressor Required for Maintenance of Mitochondrial Integrity and Metabolism during Stress, Cancer Cell 17 (2010) 41-52.
[82] L.W. Finley, A. Carracedo, J. Lee, A. Souza, A. Egia, J. Zhang, J. Teruya-Feldstein, P.I. Moreira, S.M. Cardoso, C.B. Clish, P.P. Pandolfi, M.C. Haigis, SIRT3 Opposes Reprogramming of Cancer Cell Metabolism through HIF1alpha Destabilization, Cancer cell 19 (2011) 416-428.
[83] E.L. Bell, B.M. Emerling, S.J. Ricoult, L. Guarente, SirT3 suppresses hypoxia inducible factor 1alpha and tumor growth by inhibiting mitochondrial ROS production, Oncogene (2011).
[84] M. Porcu, A. Chiarugi, The emerging therapeutic potential of sirtuin-interacting drugs: from cell death to lifespan extension, Trends Pharmacol Sci 26 (2005) 94-103.

[85] H. Ota, E. Tokunaga, K. Chang, M. Hikasa, K. Iijima, M. Eto, K. Kozaki, M. Akishita, Y. Ouchi, M. Kaneki, Sirt1 inhibitor, Sirtinol, induces senescence-like growth arrest with attenuated Ras-MAPK signaling in human cancer cells, Oncogene 25 (2006) 176-185.
[86] B. Heltweg, T. Gatbonton, A.D. Schuler, J. Posakony, H. Li, S. Goehle, R. Kollipara, R.A. Depinho, Y. Gu, J.A. Simon, A. Bedalov, Antitumor activity of a small-molecule inhibitor of human silent information regulator 2 enzymes, Cancer Res 66 (2006) 4368-4377.
[87] A. Balcerczyk, L. Pirola, Therapeutic potential of activators and inhibitors of sirtuins, BioFactors 36 (2010) 383-393.
[88] P. Signorelli, R. Ghidoni, Resveratrol as an anticancer nutrient: molecular basis, open questions and promises, J Nutr Biochem 16 (2005) 449-466.
[89] D.K. Das, S. Mukherjee, D. Ray, Resveratrol and red wine, healthy heart and longevity, Heart Fail Rev 15 (2010) 467-477.
[90] S. Rayalam, J.Y. Yang, S. Ambati, M.A. Della-Fera, C.A. Baile, Resveratrol induces apoptosis and inhibits adipogenesis in 3T3-L1 adipocytes, Phytother Res 22 (2008) 1367-1371.
[91] S. Mukherjee, D. Ray, I. Lekli, I. Bak, A. Tosaki, D.K. Das, Effects of Longevinex (modified resveratrol) on cardioprotection and its mechanisms of action, Can J Physiol Pharmacol 88 (2010) 1017-1025.
[92] J. Dudley, S. Das, S. Mukherjee, D.K. Das, Resveratrol, a unique phytoalexin present in red wine, delivers either survival signal or death signal to the ischemic myocardium depending on dose, J Nutr Biochem 20 (2009) 443-452.
[93] M. Athar, J.H. Back, X. Tang, K.H. Kim, L. Kopelovich, D.R. Bickers, A.L. Kim, Resveratrol: a review of preclinical studies for human cancer prevention, Toxicol Appl Pharmacol 224 (2007) 274-283.
[94] A. Bishayee, Cancer prevention and treatment with resveratrol: from rodent studies to clinical trials, Cancer prevention research 2 (2009) 409-418.
[95] A.L. Watters, J.B. Epstein, M. Agulnik, Oral complications of targeted cancer therapies: a narrative literature review, Oral oncology 47 (2011) 441-448.
[96] J.A. Bonner, P.M. Harari, J. Giralt, N. Azarnia, D.M. Shin, R.B. Cohen, C.U. Jones, R. Sur, D. Raben, J. Jassem, R. Ove, M.S. Kies, J. Baselga, H. Youssoufian, N. Amellal, E.K. Rowinsky, K.K. Ang, Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck, The New England journal of medicine 354 (2006) 567-578.
[97] E.E. Cohen, D.W. Davis, T.G. Karrison, T.Y. Seiwert, S.J. Wong, S. Nattam, M.F. Kozloff, J.I. Clark, D.H. Yan, W. Liu, C. Pierce, J.E. Dancey, K. Stenson, E. Blair, A. Dekker, E.E. Vokes, Erlotinib and bevacizumab in patients with recurrent or metastatic squamous-cell carcinoma of the head and neck: a phase I/II study, The lancet oncology 10 (2009) 247-257.
[98] C. Phalon, D.D. Rao, J. Nemunaitis, Potential use of RNA interference in cancer therapy, Expert Rev Mol Med 12 (2010) e26.
[99] M.R. Acharya, A. Sparreboom, J. Venitz, W.D. Figg, Rational development of histone deacetylase inhibitors as anticancer agents: a review, Mol Pharmacol 68 (2005) 917-932.
[100] A.M. Gillenwater, M. Zhong, R. Lotan, Histone deacetylase inhibitor suberoylanilide hydroxamic acid induces apoptosis through both mitochondrial and Fas (Cd95) signaling in head and neck squamous carcinoma cells, Molecular cancer therapeutics 6 (2007) 2967-2975.
[101] T. Katsura, S. Iwai, Y. Ota, H. Shimizu, K. Ikuta, Y. Yura, The effects of trichostatin A on the oncolytic ability of herpes simplex virus for oral squamous cell carcinoma cells, Cancer gene therapy 16 (2009) 237-245.
[102] T. Sato, M. Suzuki, Y. Sato, S. Echigo, H. Rikiishi, Sequence-dependent interaction between cisplatin and histone deacetylase inhibitors in human oral squamous cell carcinoma cells, Int J Oncol 28 (2006) 1233-1241.

[103] K.R. Patel, E. Scott, V.A. Brown, A.J. Gescher, W.P. Steward, K. Brown, Clinical trials of resveratrol, Annals of the New York Academy of Sciences 1215 (2011) 161-169.
[104] http://www.clinicaltrials.gov/ct2/results?term=resveratrol, (Accessed April 25, 2011).

## CHAPTER II

## SIRTUIN-3 (SIRT3), A NOVEL POTENTIAL THERAPEUTIC TARGET FOR ORAL CANCER


### ABSTRACT

**BACKGROUND:** Several sirtuin family members (SIRT1-7), evolutionarily conserved NAD-dependant deacetylases, play an important role in carcinogenesis. However, their role in oral cancer has not yet been investigated. Therefore, the aim of this study was to investigate whether sirtuins play a role in oral cancer carcinogenesis. **METHODS:** The expression levels of all sirtuins in several oral squamous cell carcinoma (OSCC) cell lines compared with normal human oral keratinocytes, and found SIRT3 was highly expressed. Therefore, tissue microarrays were used to evaluate the clinical relevance of this overexpression. SIRT3 downregulation in OSCC cell proliferation and survival was investigated and analyzed by cell proliferation and cell viability assays. Ionizing radiation and cisplatin were used to investigate whether SIRT3 downregulation can increase the sensitivity of OSCC to both treatments. To further assess the *in vivo* role of SIRT3 in OSCC carcinogenesis, we used a floor-of-mouth oral cancer murine model to study the effect of SIRT3 downregulation on OSCC tumor growth in immunodeficient mice. **RESULTS:** We show for the first time that SIRT3 is overexpressed in OSCC *in vitro* and *in vivo*, compared with other sirtuins. Downregulation of SIRT3 inhibited OSCC cell growth and proliferation, and increased its sensitivity to radiation and cisplatin treatments *in vitro*. SIRT3 downregulation reduced tumor burden *in vivo*. **CONCLUSIONS:** Our findings reveal a novel role for SIRT3 in oral cancer carcinogenesis as a promoter of cell proliferation and survival, thus implicating SIRT3 as a new potential therapeutic target to treat oral cancer.

## Introduction

Oral cancer is the eighth most common cancer worldwide, and oral squamous cell carcinoma (OSCC) accounts for more than 90% of all oral malignancies [1]. The 5-year survival rates range from 34- 62.9%, which have not changed for decades [2] . This underscores the need for new therapeutic targets to treat oral cancer. Recently, several members of the sirtuin family (SIRT1-7), the human homologues of the Sir2 gene in yeast, were found to play an important role in carcinogenesis [3]. Sirtuins function as either NAD-dependant deacetylases or ADP-ribosyl transferases, which explains their involvement in a diversity of cellular functions, including regulation of oxidative stress, increasing genomic stability, cell survival, development, metabolism, aging and longevity [4, 5]. SIRT1, the best characterized family member, targets several key regulators that affect carcinogenesis, such as the tumor suppressor p53, the DNA repair protein Ku70, and the pro-apoptotic protein FOXO [3, 6]. Current literature supports a pro-survival role for SIRT1 in colon, breast and lung cancer, through one or more of these previously mentioned targets [3]. In contrast, other reports support that SIRT1 may act as a tumor suppressor [7, 8].

Although much is known about SIRT1, less is known about other mammalian Sir2 homologues such as SIRT3. SIRT3, the only member linked to longevity in humans [9-11], is a mitochondrial protein [12-18], that is overexpressed and associated with node-positive breast cancer [19]. Moreover, during stress, SIRT3 levels increase, protecting cells from apoptosis. Thus, like SIRT1, SIRT3 may bind and deacetylate Ku70, promoting Ku70-Bax interactions and attenuating apoptosis in cardiomyocytes [20]. In addition, mitochondrial SIRT3 is required for Nampt, a stress and diet-responsive regulator of mitochondrial $NAD^+$, to promote cell survival during genotoxicity [21]. More recently, SIRT3 was found to abrogate p53 activity, thus preventing growth arrest and senescence in bladder carcinoma cells [22]. These findings suggest a role for SIRT3 in carcinogenesis, however, the role of sirtuins has not been investigated in oral cancer. Therefore, the aim of this study was to elucidate the role of sirtuins in oral cancer.

**Materials and Methods**

**Cell lines and culture.** Human OSCC cell line HSC-3 was provided by Randy Kramer (University of California, San Francisco). OSCC cell lines, UM-SCC-1 and UM-SCC-17B were from Tom Carey (University of Michigan). Primary human keratinocytes were derived from normal gingival tissues discarded from periodontal surgical procedures and approved by the University of Michigan IRB. OSCC cells were maintained in DMEM (Gibco) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin. Primary human oral keratinocytes were maintained in EpiLife medium (Cascade Biologics).

**Transient transfection.** Cells were transiently transfected with small interfering RNA (siRNA, 150 nM, a pool of 3 target-specific siRNA) against SIRT3 or a non-targeting control (Santa Cruz Biotechnology), in serum-free medium containing Lipofectamine Plus (Invitrogen). Transfection efficiency was assessed by Western blotting.

**Stable transfection.** UM-SCC-17B cells were transduced with SIRT3-shRNA (sc61555-vs) or scrambled-shRNA (sc-108084, Santa Cruz Biotechnology) lentiviral particles in 0.5ml of serum-free media, then selected in 10 μg/ml puromycin for an additional 10 days (sc-108071, Santa Cruz Biotechnology). Surviving cell colonies were picked and propagated before testing for SIRT3 expression.

**Cell proliferation and colony formation assays.** To determine the effect of Sirtinol, NAM, ionizing radiation (IR) and cisplatin on cell proliferation, the QUANT Cell Proliferation Assay Kit was used according to manufacturer's instructions (Invitrogen). For colony formation assays, OSCC cells were transfected as mentioned above and cultured for one week. Colonies were stained with 0.5% crystal violet and colonies containing greater than 50 cells were counted.

**Apoptosis cell death detection by ELISA.** To measure apoptosis *in vitro*, a DNA-fragmentation ELISA assay was used according to manufacturer's instructions (Roche Diagnostics, Indianapolis, IN, USA).

**Immunoblot analysis.** To evaluate the expression levels of sirtuins in OSCC cells compared with normal primary human keratinocytes or the transfection/transduction efficiency of cells, cells were treated as described above or in figure legends, washed once with PBS, and lysed in RIPA lysis buffer (R0278, Sigma) that contained 1% protease inhibitor cocktail (P8340, Sigma) on ice for 30 min. Lysates were adjusted for protein concentration with the BCA protein assay kit (Bio Rad). Lysate proteins were resolved by SDS-PAGE and transferred to Immobilon-P membranes (Millipore). Western blotting was performed with primary antibodies and horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibodies and developed with the ECL-Plus detection system (Pierce). Antibodies for SIRT1 (sc-15404) and SIRT2 (sc-20966) were from Santa Cruz Biotechnology. The SIRT3 antibody (2627) was from Cell Signaling. Antibodies to SIRT4 (IMG-3580), SIRT5 (IMG-479), and SIRT7 (IMG-425) were from IMGENEX. The SIRT6 antibody (AP6245a) was obtained from ABGENT. To demonstrate equal protein loading, membranes were stripped and reprobed with an anti-β-actin antibody (sc-1615, Santa Cruz Biotechnology).

**Tissue Microarrays.** Immunohistochemical analyses were performed to determine the expression of SIRT3 and SIRT7 in human normal and OSCC tissues, using OSCC tissue microarrays (OR601 and HN241, Biomax.us) and the histostatin kit (95-6143, Zymed lab) as per the manufacturer's instructions. Antibodies to SIRT3 (AP6242a) and SIRT7 (AP6246a) were obtained from ABGENT. Staining intensities were graded in a blinded manner as low or high by a pathologist.

**Immunodeficient mouse model of human head and neck squamous cell carcinoma.** Three-week-old athymic nude mice (NCr-nu/nu strain; NCI, Frederick, MD, USA), that weighed 20 and 25 g were anesthetized by intraperitoneal injection with 100 mg/kg ketamine and 10 mg/kg xylazine. The human

OSCC cell line UM-SCC-17B stably expressing SIRT3-shRNA or scrambled-shRNA was grown to 70% confluence prior to injection. We used a murine floor-of-mouth model, which we previously optimized to produce 4-5 mm tumors, corresponding to a palpable tumor volume of 35-60 $mm^3$, within ~2-4 weeks post-injection [23]. In brief, cells were suspended in DMEM, chilled on ice, and resuspended in an equal volume of growth factor reduced Matrigel (BD Biosciences, San Jose, CA, USA; Cat #354230), to a final concentration of 2.5 x $10^5$/ 0.1 ml. The cell/matrigel solution was chilled on ice prior to injection, and each animal was injected with an equal number of OSCC cells (2.5 x $10^5$) submucosally in the floor of the mouth. After three weeks, mice were euthanized and digital caliper was used to determine the tumor volume using the formula: $a$ x $a$ x $b$/2, where $a$ is the smaller dimension.

**Statistical analysis.** Values were expressed as means ± SD. Intergroup differences were determined by two-way analysis of variance (ANOVA) and Scheffe's multiple-comparison test. Statistical significance was defined as $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$. For tissue microarray analyses a chi-square test was employed. For the *in vivo* studies, independent *t*- test with unequal variances was used. All experiments were repeated at least three times.

**Results**

**SIRT3 is overexpressed in oral squamous cell carcinomas.** To determine whether sirtuins play a role in OSCC, we examined the protein levels of all sirtuins (SIRT1-7) in several OSCC cell lines (HSC-3, UM-SCC-1 & UM-SCC-17B) and compared those cells with normal primary human oral keratinocytes (Fig. 2.1A). Only SIRT3, and to a lesser extent SIRT7, were overexpressed in all three cell lines compared with primary keratinocytes. To further examine the *in vivo* and clinical relevance of SIRT3 and SIRT7, immunohistochemical analyses were performed for both sirtuins using tissue microarrays of OSCCs (Fig. 2.1 and Fig. 2.S1). A total of 52 samples, containing 42 malignant and 10 normal tissue samples were analyzed. Grade 1, 2, &3 tumors from the tongue, cheek, gingiva, lip and oral mucosa were analyzed along with normal tissue from the tongue, palate, and gingiva (Table 2.1 and Table 2.S1). Staining

intensity was assessed as low or high (Table 2.1 and Table 2.S1). SIRT3 expression was significantly higher in OSCC tissues compared with normal tissues (Fig. 2.1B; Table 2.1, $^{*}p \leq 0.05$), whereas SIRT7 expression levels were not significantly different (Fig. 2.S1A; Table 2.S1, $p \geq 0.05$). SIRT3 staining intensity data from Table 1 were graphically illustrated (Fig. 2.1C). SIRT3 exhibited an opposite pattern of expression between OSCC and normal tissues (Fig. 2.1C, *top*). Furthermore, because the tongue accounts for 30% of oral malignancies [1], we specifically examined tongue samples separately. SIRT3 staining intensity was significantly higher in OSCC tongue samples compared with normal tongue tissues (Fig. 2.1C, *bottom*; Table 2.1, $^{*}p \leq 0.04$).

**Sirtuin inhibitors, Sirtinol and NAM, inhibit cell growth and proliferation, and induce apoptosis.** Having established that sirtuins, and specifically SIRT3 was associated with OSCC, we next explored the role of sirtuins in modulating OSCC cell growth and proliferation. To this end, we tested the commonly used sirtuin inhibitors, sirtinol and nicotinamide (NAM), which inhibited cell growth in breast and lung cancers [24, 25]. Both inhibitors inhibited cell growth and proliferation in OSCC cells (Fig. 2.2A). In addition, both inhibitors induced apoptosis in OSCC cells compared with untreated controls as determined by cell death ELISA assays that measure DNA fragmentation (Fig. 2.2B).

**SIRT3 downregulation inhibits cell growth and proliferation, and promotes apoptosis in OSCC cells.** To gain further insight into the effects of SIRT3 on OSCC cell survival and proliferation, we performed colony formation assays in the context of SIRT3 inhibition with siRNA. As with cell growth studies, SIRT3 downregulation inhibited colony formation in OSCC cells compared with controls (Fig. 2.3A). Furthermore, downregulation of SIRT3 also promoted apoptosis in OSCC cells, mimicking the effect of Sirtinol and NAM on these cells (Fig. 2.3B).

**SIRT3 downregulation enhances the sensitivity of OSCC cells to radiation and cisplatin-induced cytotoxicity.** Because radiation is the primary treatment modality in head and neck cancer, and cisplatin

is one of the first chemotherapeutic drugs used to treat OSCC [26], we asked whether SIRT3 downregulation with siRNA enhances the sensitivity of OSCC cells to radiation and cisplatin. Cytotoxicity assays were performed first to optimize ionizing radiation (IR) and cisplatin (CDDP) doses, and determine the $IC_{50}$ for both treatments in all cell lines (data not shown). Indeed, downregulation of SIRT3 with siRNA enhanced the sensitivity of UM-SCC-1 and UM-SCC-17B cell lines to both treatments compared with untreated controls and cells treated with radiation or cisplatin alone (Fig. 2.4).

**SIRT3 downregulation reduces OSCC tumor burden *in vivo*.** To further demonstrate the role of SIRT3 in OSCC carcinogenesis *in vivo*, we used a murine floor-of-mouth model that mimics human OSCC [23, 27]. The UM-SCC-17B cell line was selected for examination in this model because of its highly aggressive nature, resistance to radiation therapy, and because it commonly produces tumors in this *in vivo* model [23]. The UM-SCC-17B cell line was used to produce stable clones expressing downregulated levels of SIRT3 using SIRT3-shRNA, and scrambled-shRNA was used for controls (Fig. 2.5A). SIRT3-shRNA (clone 4) and Scrambled-shRNA (clone 1) were selected for the *in vivo* injections. In agreement with our in vitro data, our mouse model data show that downregulation of SIRT3 in OSCC cells significantly inhibited tumor growth *in vivo* (Fig. 2.5B). Specifically, mean tumor volume for the SIRT3 downregulated group and control group was 26.948 ($mm^3$) and 112.325 ($mm^3$), respectively (Fig. 2.5B; Table 2.2, $^{**}p \leq 0.001$).

**Discussion**

We report for the first time a role for sirtuins and specifically, SIRT3 in OSCC carcinogenesis. There is an emerging role for several sirtuins in carcinogenesis [4]. However, their role in oral cancer has not been investigated. Thus, we explored their potential role in OSCC by evaluating their expression level in several OSCC cell lines compared with normal human oral keratinocytes. Our initial finding that SIRT3 was overexpressed in OSCC cells compared with normal human oral keratinocytes, led us to hypothesize that SIRT3 might play a role in OSCC carcinogenesis. Thus, to further explore the clinical

relevance of this overexpression, we used tissue microarray analyses of OSCC tissues. The high expression of SIRT3 in OSCC tissues compared with normal tissues, further supported a role for SIRT3 in oral cancer carcinogenesis. Sirtuin inhibitors, such as Sirtinol and Nicotinamide (NAM), that have been used to inhibit cell growth in several types of cancers, such as breast and lung cancers [24] also inhibited cell growth and proliferation, and induced apoptosis in several OSCC cell lines.

Although some suggest a pro-apoptotic role for SIRT3 [28-30], others suggest a pro-survival role [20-22, 31]. In addition, some reports suggest that SIRT3 is exclusively a mitochondrial protein [12-18], however, others find that SIRT3 can be found in the cytosol and nucleus under different cellular events [20, 32, 33]. These reports underscore the complexity of sirtuins' biological functions, which may differ according to their tissue of origin or cancer type. For example, SIRT1 functions in the neural system to promote neural cell survival and to protect against genomic toxicity [30], however, this may not be true in all cancer types. There is a discrepancy in the literature regarding the role of SIRT1 in cancer [7, 8, 24]. After investigating the effect of SIRT1 in several cancer types, Stunkel et al. [34] concluded that SIRT1's function in cancer is cell-context dependant, and the role of SIRT1 can be independent of its deacetylase function. This cell specificity may also be true for SIRT3. When SIRT3 is overexpressed in cardiomyocytes, it increases stress resistance and plays a protective role against cell death and apoptosis [20]. SIRT3 also seems to play a protective role against cardiac hypertrophy and heart failure [35].Furthermore, SIRT3 is overexpressed in breast cancer, and it was shown to modulate p53 activity, preventing growth arrest and senescence in bladder carcinoma cells [19, 22]. Because our present findings show that SIRT3 is overexpressed in OSCC tissues and cells (Fig. 2.1), we surmised that SIRT3 would also play a pro-survival role in oral cancer.

Our current data indicate that SIRT3 levels are elevated in head and neck cancer and suppression of SIRT3 levels reduces several tumorigenic parameters *in vitro* and *in vivo*. In contrast, a recent study speculated on the role of SIRT3 in head and neck cancer by reviewing published gene array data from another source and indicated that SIRT3 levels are decreased in head and neck cancer [36]. Those findings are contradictory to findings in the current report; however, it must be noted that those studies were

limited, since they were only gene array reviews and not a full functional assessment of SIRT3 *in vitro* and *in vivo* as in the present study.

Downregulation of SIRT3 inhibited colony formation and induced apoptosis in OSCC cells. In addition, we investigated whether SIRT3 could modulate the sensitivity of OSCC cells to both IR and cisplatin treatment. We tested UM-SCC-1 and UM-SCC-17B cells, which are both highly resistant to radiation [37, 38], and the former is also resistant to cisplatin treatment (data not shown). Our data demonstrate that SIRT3 downregulation sensitized OSCC cells to both IR and cisplatin treatment, indicating that SIRT3 is important to the modulation of OSCC induced-resistance to both treatments. Thus, targeting SIRT3 to induce OSCC cell cytotoxicity in patients that have high SIRT3 expressing tumors could be advantageous, since lower doses of treatment would be required. Furthermore, SIRT3 could serve as an adjunctive target to improve the efficacy and decrease the side effects of conventional treatments. In addition, despite SIRT3-deficient mice showing hyperacetylation of mitochondrial proteins, they are surprisingly healthy with normal bone mineral density and an unremarkable phenotype compared to SIRT3-wildtype mice [18]. This suggests that targeting SIRT3 in oral cancer may be less toxic to normal cells versus cancer cells. Parenthetically, gene deletions of other sirtuins produce more aggressive and lethal phenotypes [39, 40].

To further demonstrate the important role of SIRT3 in OSCC carcinogenesis, we used an *in vivo* murine floor-of-mouth model that mimics human OSCC [23, 27]. This model has the advantage that tumors injected into the floor-of-mouth behave like human head and neck SCC, growing in the same environment, growing aggressively and invading surrounding tissues. It is noteworthy that our data indicate that tumor cells with low levels of SIRT3 grow slower, produce smaller tumor, and have less tumor volume compared with controls (Fig. 2.3 and Table 2.1). These findings support the *in vitro* data and indicate that SIRT3 plays an important role in oral cancer carcinogenesis *in vivo*.

In summary, our findings reveal a novel role for SIRT3 in oral cancer carcinogenesis as a modulator of cell proliferation and survival, supported by *in vitro* and *in vivo* data. This implicates SIRT3 as a new potential therapeutic target for treating oral cancer.

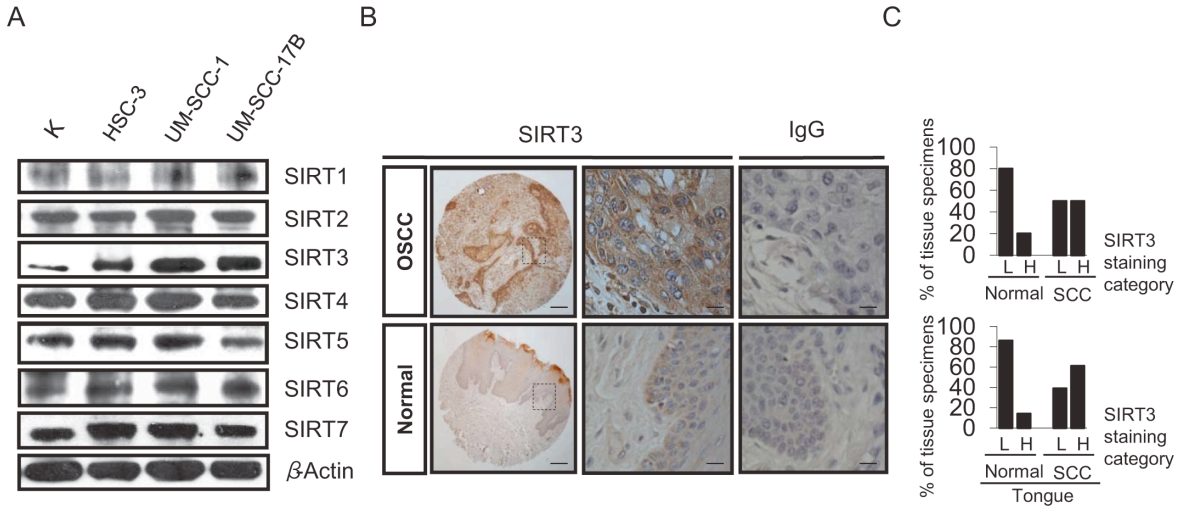


**Figure 2.1: SIRT3 is overexpressed in oral squamous cell carcinoma.** (A) Immunoblots reveal the levels of sirtuins (SIRT1-7) in OSCC cell lines (HSC-3, UM-SCC-1 and UM-SCC-17B), and in normal human oral keratinocytes (K). β-Actin served as loading control. (B) These representative samples show (*top*) SIRT3 expression levels in OSCC (tongue), and (*bottom)* in normal tissues. Immunoglobulin G (IgG) served as negative control. Scale bars = 200 µm for low-magnifications photomicrographs (left column); 50 µm for high-magnifications photomicrographs (middle and right columns) (C) These charts illustrate the percentage of normal and OSCC tissue specimens that expressed SIRT3 (*top*) in all samples and (*bottom*) in tongue samples, as determined by immunohistochemical staining. Note that SIRT3 staining intensities were designated as LOW (L) or HIGH (H) from Table 2.1.

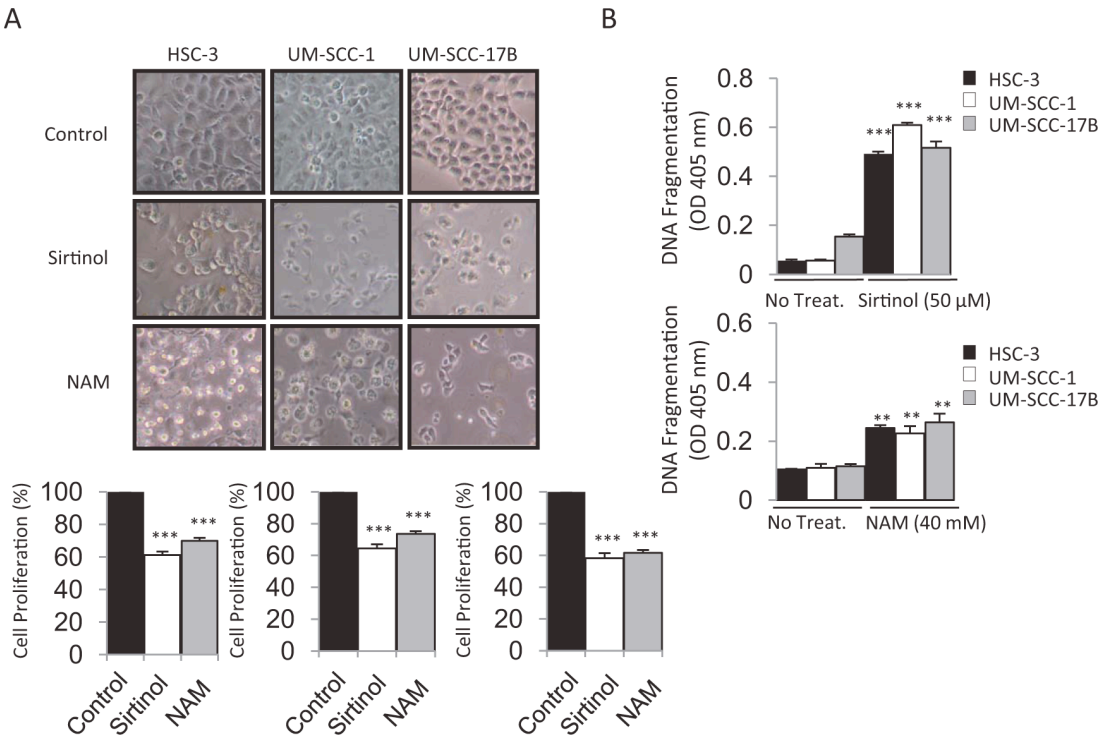


**Figure 2.2: The sirtuin inhibitors, sirtinol and nicotinamide (NAM) inhibit cell growth and proliferation, and induce apoptosis.** (A) Shown are (*top*) phase-contrast images, and (*bottom*) histograms that reveal the morphology of cell growth and cell proliferation (%) of HSC-3, UM-SCC-1 and UM-SCC-17B oral squamous cell carcinoma (OSCC) cell lines after treatment with sirtuin inhibitors, sirtinol (50 µM), and nicotinamide (NAM, 40 mM) for 16 and 24 h, respectively (Original magnification x100). (B) Cell death-detection ELISA assays were used to measure DNA fragmentation in cells after treatment with (*top*) sirtinol and *(bottom)* NAM as indicated. ** $p \leq 0.01$; *** $p \leq 0.001$; OD, optical density.

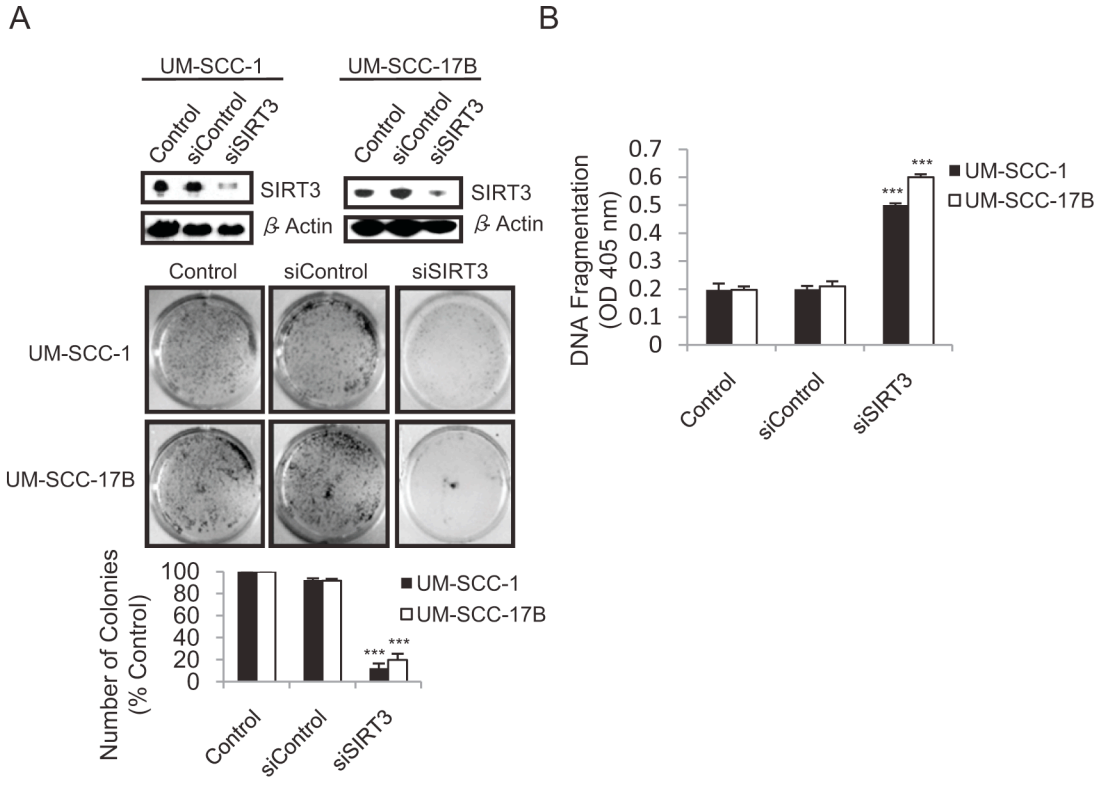


**Figure 2.3: Sirtuin-3 (SIRT3) downregulation inhibits cell growth and proliferation and promotes apoptosis in oral squamous cell carcinoma (OSCC) cells.** (A) (*top*) Immunoblots reveal the transfection efficiency of SIRT3 in the UM-SCC-1 and UM-SCC17B OSCC cell lines at 36 hours after transfection with SIRT3 small interfering RNA (siSIRT3) or non-targeting control (siControl) (150 nM). β-Actin served as loading control. (*middle*) Cells were transfected as indicated and were cultured for one week, then stained with crystal violet and photographed. (*bottom)* Numbers of colonies are presented as the percentage of colonies obtained relative to controls. (B) Cell death- detection ELISA assays were used to measure DNA fragmentation in the cells after transfection. $^{**}$ $p \leq 0.01$; $^{***}$ $p \leq 0.001$; OD, optical density.

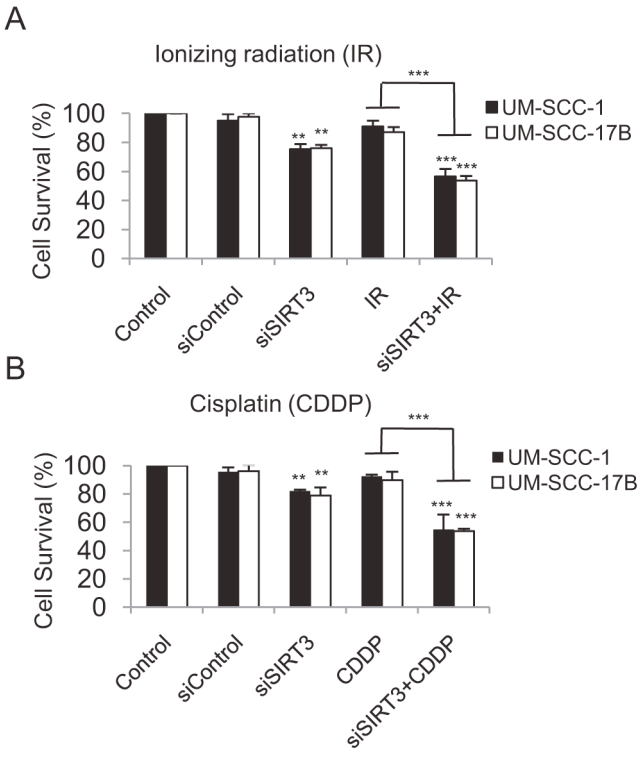


**Figure 2.4: Sirtuin-3 (SIRT3) downregulation enhances the sensitivity of oral squamous cell carcinoma (OSCC) to radiation and cisplatin-induced cytotoxicity.** (A) OSCC cells (UM-SCC-1 and UM-SCC-17B) were either untransfected or transfected with small interfering SIRT3 (siSIRT3) (150 nM) and were treated with (A) ionizing radiation (2.5 Gy) or (B) cisplatin (20 μM) for 24 h, and cytotoxicity was determined by the QUANT Cell Proliferation Assay Kit (Invitrogen) $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

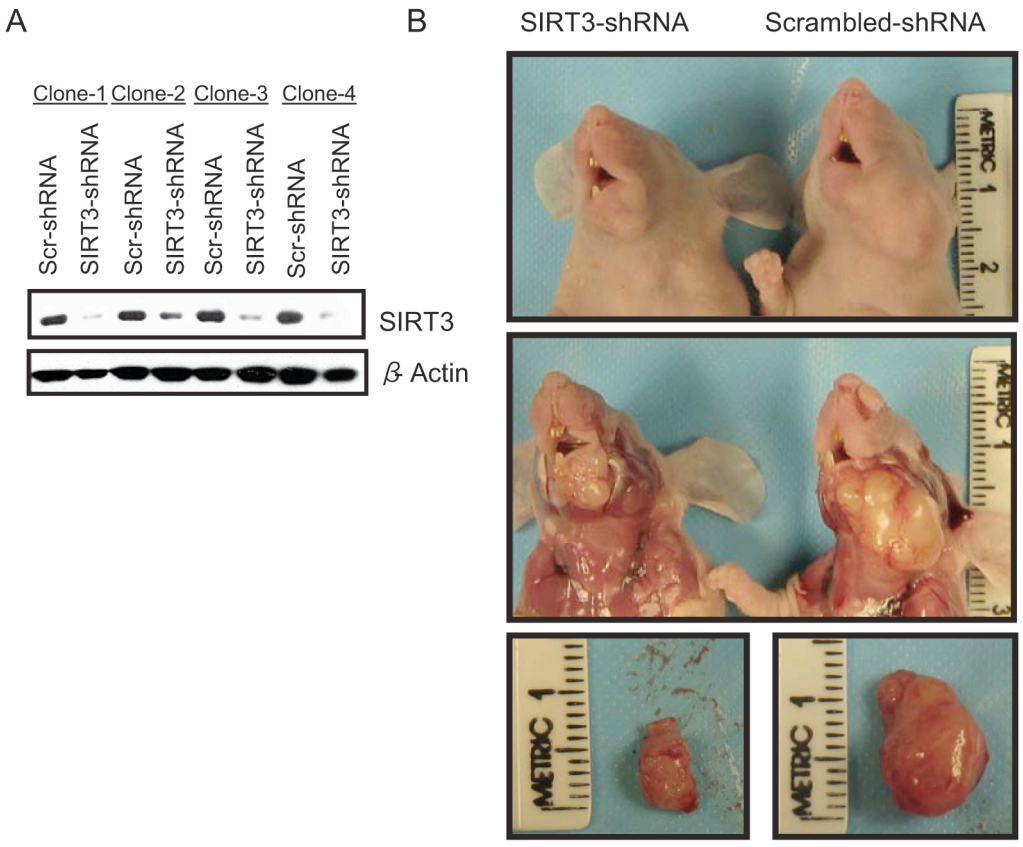


**Figure 2.5: Sirtuin-3 (SIRT3) downregulation reduces oral squamous cell carcinoma (OSCC) tumor burden *in vivo*.** (A) These immunoblots show SIRT3 expression levels in 4 clones from UM-SCC-17B cell lines where stably transduced with scrambled-short hairpin RNA (Scr-shRNA) or SIRT3-shRNA after 10 days of selection using Puromycin. β-Actin served as loading control. (B) Mice were injected with OSCC cells that stably Scr-shRNA or SIRT3-shRNA. Images show *(top)* the superficial growth of tumors in the head and neck region of the mice, *(middle)* dissected tumors in situ, and *(bottom)* dissected and isolated tumors for the 2 groups.

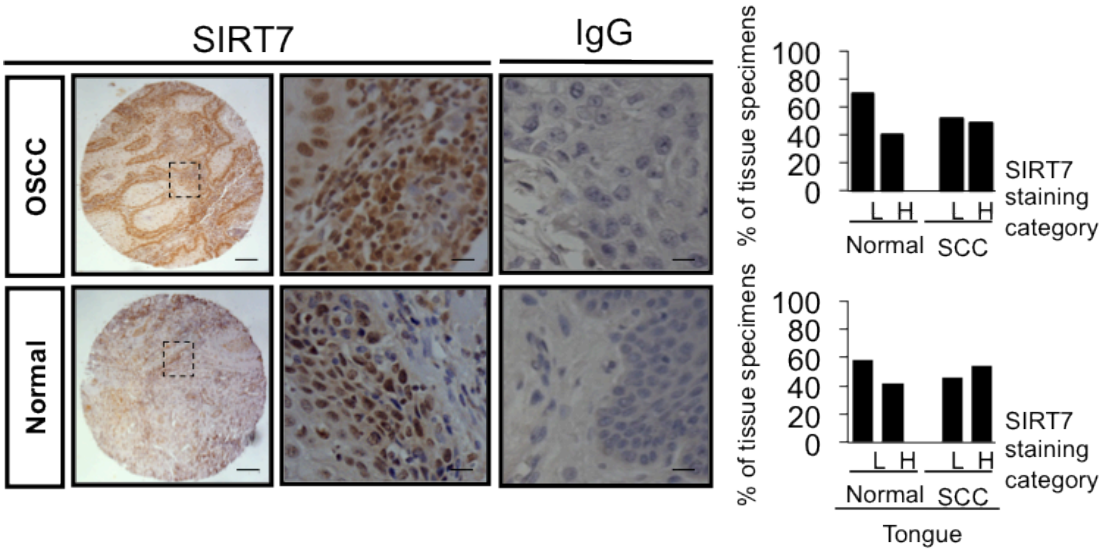


**Figure 2.S1: The expression levels of SIRT7 in OSCCs.** (A) Representative samples showing SIRT7 expression levels in OSCC (tongue) (*top)*, and normal tissues (*bottom)*. IgG served as negative control. (B) Percentage of normal and OSCC tissue specimens expressing SIRT7 in general (*top*) and in tongue (*bottom*), as determined by immunohistochemical staining. Note that SIRT7 staining intensity was designated as category Low (L) or HIGH (H) from supplementary Table 2.S1 data. (Scale bar represents 200 and 50 μm in low and high magnifications, respectively).

**Table 2.1: Correlation of SIRT3 expression and clinicopathological variables in normal and OSCC tissues**

| | No. of tissue specimens | SIRT3 staining intensity No. of tissue specimens (%) | | *P* value |
|---|---|---|---|---|
| | | **Low** | **High** | |
| Normal | 10 | 8 (80) | 2 (20) | |
| Tumor | 42 | 21 (50) | 21 (50) | Normal vs Tumor 0.05* |
| **Normal** | 10 | | | |
| Tongue | 7 | 6 (86) | 1 (14) | |
| **Tumor** | 42 | | | |
| Tongue | 26 | 10 (39) | 16 (61) | Normal Tongue vs Tumor Tongue 0.04* |

Statistical analysis: Chi-square test; *significant difference $p \leq 0.05$

**Table 2.2: Summary of tumor volume in mice injected with UM-SCC-17B cells stably expressing Scrambled-shRNA or SIRT3-shRNA**

| | Scrambled-shRNA | SIRT3-shRNA |
|---|---|---|
| Animal | Tumor Volume ($mm^3$) | Tumor Volume ($mm^3$) |
| 1 | 190.8 | 21.1 |
| 2 | 186.9 | 35.75 |
| 3 | 100.7 | 32.8 |
| 4 | 165.1 | 20.1 |
| 5 | 124.62 | 26 |
| 6 | 92.23 | 28.5 |
| 7 | 71.3 | 37.4 |
| 8 | 64.4 | 40.63 |
| 9 | 64 | 15.3 |
| 10 | 63.2 | 11.9 |
| Mean Volume | 112.325 | 26.948*** |

Statistical analysis: independent *t*-test with unequal variances; ***significant difference $p \leq 0.001$

**Table 2.S1: Correlation of SIRT7 expression and clinicopathological variablesin normal and oral SCC tissues**

| | No. of tissue specimens | SIRT7 staining intensity No. of tissue specimens (%) | | *P* value |
|---|---|---|---|---|
| | | **Low** | **High** | |
| Normal | 10 | 6 (60) | 4 (40) | |
| Tumor | 42 | 20 (48) | 22 (52) | Normal vs Tumor $p \leq 0.8$ |
| **Normal** | 10 | | | |
| Tongue | 7 | 4(58) | 3 (42) | |
| **Tumor** | 42 | | | |
| Tongue | 26 | 12 (46) | 14 (54) | Normal Tongue vs Tumor Tongue $p \leq .2$ |

Statistical analysis: Chi-square test; *significant difference $p \leq 0.05$

**References**


1. Bsoul SA, Huber MA, Terezhalmy GT. Squamous cell carcinoma of the oral tissues: a comprehensive review for oral healthcare providers. J Contemp Dent Pract. 2005;6: 1-16.
2. National Cancer Institute. Aailable from URL:http://seer.cancer.gov/statfacts/html/oralcav.html?statfacts_page=oralcav.html&x (accessed January 7, 2010).
3. Taylor DM, Maxwell MM, Luthi-Carter R, Kazantsev AG. Biological and potential therapeutic roles of sirtuin deacetylases. Cell Mol Life Sci. 2008;65: 4000-4018.
4. Saunders LR, Verdin E. Sirtuins: critical regulators at the crossroads between cancer and aging. Oncogene. 2007;26: 5489-5504.
5. Haigis MC, Guarente LP. Mammalian sirtuins--emerging roles in physiology, aging, and calorie restriction. Genes Dev. 2006;20: 2913-2921.
6. Brunet A, Sweeney LB, Sturgill JF, et al. Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. Science. 2004;303: 2011-2015.
7. Fu M, Liu M, Sauve AA, et al. Hormonal control of androgen receptor function through SIRT1. Mol Cell Biol. 2006;26: 8122-8135.
8. Wang RH, Sengupta K, Li C, et al. Impaired DNA damage response, genome instability, and tumorigenesis in SIRT1 mutant mice. Cancer Cell. 2008;14: 312-323.
9. Bellizzi D, Dato S, Cavalcante P, et al. Characterization of a bidirectional promoter shared between two human genes related to aging: SIRT3 and PSMD13. Genomics. 2007;89: 143-150.
10. Bellizzi D, Rose G, Cavalcante P, et al. A novel VNTR enhancer within the SIRT3 gene, a human homologue of SIR2, is associated with survival at oldest ages. Genomics. 2005;85: 258-263.
11. Rose G, Dato S, Altomare K, et al. Variability of the SIRT3 gene, human silent information regulator Sir2 homologue, and survivorship in the elderly. Exp Gerontol. 2003;38: 1065-1070.
12. Cooper HM, Spelbrink JN. The human SIRT3 protein deacetylase is exclusively mitochondrial. Biochem J. 2008;411: 279-285.
13. Schwer B, North BJ, Frye RA, Ott M, Verdin E. The human silent information regulator (Sir)2 homologue hSIRT3 is a mitochondrial nicotinamide adenine dinucleotide-dependent deacetylase. J Cell Biol. 2002;158: 647-657.
14. Chen XJ, Clark-Walker GD. sir2 mutants of Kluyveromyces lactis are hypersensitive to DNA-targeting drugs. Mol Cell Biol. 1994;14: 4501-4508.
15. Michishita E, Park JY, Burneskis JM, Barrett JC, Horikawa I. Evolutionarily conserved and nonconserved cellular localizations and functions of human SIRT proteins. Mol Biol Cell. 2005;16: 4623-4635.
16. Shi T, Wang F, Stieren E, Tong Q. SIRT3, a mitochondrial sirtuin deacetylase, regulates mitochondrial function and thermogenesis in brown adipocytes. J Biol Chem. 2005;280: 13560-13567.
17. Onyango P, Celic I, McCaffery JM, Boeke JD, Feinberg AP. SIRT3, a human SIR2 homologue, is an NAD-dependent deacetylase localized to mitochondria. Proc Natl Acad Sci U S A. 2002;99: 13653-13658.
18. Lombard DB, Alt FW, Cheng HL, et al. Mammalian Sir2 homolog SIRT3 regulates global mitochondrial lysine acetylation. Mol Cell Biol. 2007;27: 8807-8814.
19. Ashraf N, Zino S, Macintyre A, et al. Altered sirtuin expression is associated with node-positive breast cancer. Br J Cancer. 2006;95: 1056-1061.
20. Sundaresan NR, Samant SA, Pillai VB, Rajamohan SB, Gupta MP. SIRT3 is a stress-responsive deacetylase in cardiomyocytes that protects cells from stress-mediated cell death by deacetylation of Ku70. Mol Cell Biol. 2008;28: 6384-6401.
21. Yang H, Yang T, Baur JA, et al. Nutrient-Sensitive Mitochondrial NAD(+) Levels Dictate Cell Survival. Cell. 2007;130: 1095-1107.
22. Li S, Banck M, Mujtaba S, Zhou MM, Sugrue MM, Walsh MJ. p53-Induced growth arrest is regulated by the mitochondrial SirT3 deacetylase. PLoS ONE. 2010;5: e10486.
23. Henson B, Li F, Coatney DD, et al. An orthotopic floor-of-mouth model for locoregional growth and spread of human squamous cell carcinoma. J Oral Pathol Med. 2007;36: 363-370.
24. Ota H, Tokunaga E, Chang K, et al. Sirt1 inhibitor, Sirtinol, induces senescence-like growth arrest with attenuated Ras-MAPK signaling in human cancer cells. Oncogene. 2006;25: 176-185.
25. Luo J, Nikolaev AY, Imai S, et al. Negative control of p53 by Sir2alpha promotes cell survival under stress. Cell. 2001;107: 137-148.
26. Bauer JA, Trask DK, Kumar B, et al. Reversal of cisplatin resistance with a BH3 mimetic, (-)-gossypol, in head and neck cancer cells: role of wild-type p53 and Bcl-xL. Mol Cancer Ther. 2005;4: 1096-1104.

27. Wolter KG, Wang SJ, Henson BS, et al. (-)-gossypol inhibits growth and promotes apoptosis of human head and neck squamous cell carcinoma in vivo. Neoplasia. 2006;8: 163-172.
28. Marfe G, Tafani M, Indelicato M, et al. Kaempferol induces apoptosis in two different cell lines via Akt inactivation, Bax and SIRT3 activation, and mitochondrial dysfunction. J Cell Biochem. 2009;106: 643-650.
29. Allison SJ, Milner J. SIRT3 is pro-apoptotic and participates in distinct basal apoptotic pathways. Cell Cycle. 2007;6: 2669-2677.
30. Schild D. Suppression of a new allele of the yeast RAD52 gene by overexpression of RAD51, mutations in srs2 and ccr4, or mating-type heterozygosity. Genetics. 1995;140: 115-127.
31. Benigni A, Corna D, Zoja C, et al. Disruption of the Ang II type 1 receptor promotes longevity in mice. J Clin Invest. 2009;119: 524-530.
32. Scher MB, Vaquero A, Reinberg D. SirT3 is a nuclear NAD+-dependent histone deacetylase that translocates to the mitochondria upon cellular stress. Genes Dev. 2007;21: 920-928.
33. Nakamura Y, Ogura M, Tanaka D, Inagaki N. Localization of mouse mitochondrial SIRT proteins: shift of SIRT3 to nucleus by co-expression with SIRT5. Biochem Biophys Res Commun. 2008;366: 174-179.
34. Stunkel W, Peh BK, Tan YC, et al. Function of the SIRT1 protein deacetylase in cancer. Biotechnol J. 2007;2: 1360-1368.
35. Pillai VB, Sundaresan NR, Kim G, et al. Exogenous NAD blocks cardiac hypertrophic response via activation of the SIRT3-LKB1-AMP-activated kinase pathway. J Biol Chem. 2010;285: 3133-3144.
36. Kim HS, Patel K, Muldoon-Jacobs K, et al. SIRT3 Is a Mitochondria-Localized Tumor Suppressor Required for Maintenance of Mitochondrial Integrity and Metabolism during Stress. Cancer Cell. 2010;17: 41-52.
37. Grenman R, Burk D, Virolainen E, Wagner JG, Lichter AS, Carey TE. Radiosensitivity of head and neck cancer cells in vitro. A 96-well plate clonogenic cell assay for squamous cell carcinoma. Arch Otolaryngol Head Neck Surg. 1988;114: 427-431.
38. Carey TE, Van Dyke DL, Worsham MJ, et al. Characterization of human laryngeal primary and metastatic squamous cell carcinoma cell lines UM-SCC-17A and UM-SCC-17B. Cancer Res. 1989;49: 6098-6107.
39. Sequeira J, Boily G, Bazinet S, et al. sirt1-null mice develop an autoimmune-like condition. Exp Cell Res. 2008;314: 3069-3074.
40. Lombard DB, Schwer B, Alt FW, Mostoslavsky R. SIRT6 in DNA repair, metabolism and ageing. J Intern Med. 2008;263: 128-141.

# CHAPTER III

## RECEPTOR INTERACTING PROTEIN (RIP) AND SIRTUIN-3 (SIRT3) ARE ON OPPOSITE SIDES OF ANOIKIS AND TUMORIGENESIS


### ABSTRACT

**BACKGROUND:** Regulating crosstalk between anoikis and survival signaling pathways is crucial to regulating tissue processes and mitigating diseases like cancer. Previously, we showed that anoikis activates a CD95/Fas-mediated signaling pathway regulated by receptor-interacting protein (RIP), a kinase that shuttles between Fas-mediated cell death and integrin/FAK-mediated survival pathways. Since sirtuin-3 (SIRT3), an NAD-dependent deacetylase, is known to regulate cell survival, metabolism, and tumorigenesis, we hypothesized that SIRT3 might engage in crosstalk with Fas/RIP/integrin/FAK survival-death pathways in cancer cell systems. **METHODS:** Using immunohistochemical staining, immunoblotting, human tissue microarrays, and overexpression and suppression approaches *in vitro* and *in vivo* we examined the roles of RIP and SIRT3 in oral squamous cell carcinoma (OSCC) anoikis resistance and tumorigenesis. **RESULTS:** RIP and SIRT3 have an opposite expression profile in OSCC cells and tissues. Stable suppression of RIP enhances SIRT3 levels, whereas, stable suppression of SIRT3 does not impact RIP levels in OSCC cells. As OSCC cells become anoikis-resistant they form multicellular aggregates or oraspheres in suspension conditions, and their expression of SIRT3 increases as their RIP expression decreases. Also, anoikis-resistant OSCC cells with higher SIRT3 and low RIP expression induce an increased tumor burden and incidence in mice unlike their adherent OSCC cell counterparts. Furthermore, stable suppression of SIRT3 inhibits anoikis resistance and reduces tumor incidence. **CONCLUSION:** RIP is a likely upstream negative regulator of SIRT3 in anoikis resistance, and an anoikis-resistant orasphere phenotype defined by higher SIRT3 and low RIP expression contributes to a more aggressive phenotype in OSCC development.

## Introduction

Anoikis—apoptotic cell death triggered by loss of extracellular matrix (ECM) contacts— is dysregulated in many chronic debilitating and fatal diseases. Cancer cells evade apoptosis and possess self-sufficiency in growth signals; two important hallmarks of cancer cells[1]. Thus, cancer cells can evade apoptosis by escaping anoikis and becoming anoikis resistant. Anoikis-resistance or anchorage-independent growth contributes to cancer development and progression[2-5]. Although smoking, alcohol consumption, and HPV are risk factors for oral cancer, other factors contributing to tumorigenicity are poorly studied. One such factor, anoikis-resistance induces more aggressive tumors in oral squamous cell carcinoma (OSCC)[6-8].

Oral cancer is one of the leading causes of death worldwide, and oral squamous cell carcinoma (OSCC) accounts for more than 90% of oral malignancies[9], yet survival rates for oral cancer have not improved in decades. These disheartening statistics underscore the need to examine its pathogenesis and to identify novel biomarkers and modes of therapy.

We recently showed that receptor interacting protein (RIP), shuttles between CD95/Fas death and FAK survival signaling pathways to mediate anoikis in OSCC cells[10]. Hence, under anoikis conditions, FAK and RIP dissociate, leading to the association of RIP with Fas and the formation of the death inducing signaling complex, thus enhancing apoptosis. These findings support the development of therapeutics that can target RIP as a switch to control cell death or survival pathways to ultimately regulate normal tissue processes and tumorigenesis in cancer patients.

Sirtuins (SIRT1-7), the mammalian homologues of the Sir2 gene in yeast, have an emerging role in regulating cellular processes and functions including cell survival, apoptosis, oxidative stress, development, metabolism, and aging[11, 12]. We recently reported that SIRT3, one of the mitochondrial sirtuins[13-15], is overexpressed in OSCC cells and tissues compared to normal, and that downregulation of SIRT3 in OSCC cells inhibited cell growth and proliferation, and increased their sensitivity to both

radiation and chemotherapy treatments[14]. In addition, by using a floor-of-mouth oral cancer murine model that mimics human OSCC[16, 17], we showed that SIRT3 downregulation reduced tumor burden *in vivo*, implicating a prosurvival role for SIRT3 in oral cancer tumorigenesis[14]. However, the role of SIRT3 in anoikis resistance has not been investigated. Here, we reported for the first time a role for SIRT3 in mediating anoikis-resistance in oral cancer and its potential cross-talk with RIP.

## Materials and Methods

### Cell lines and culture

Human OSCC cells (HSC-3) was kindly provided by Randy Kramer (University of California, San Francisco, CA). The human OSCC cells UM-SCC-14A was a gift from Tom Carey (University of Michigan, Ann Arbor, MI). The poorly differentiated aggressive tongue SCC cell line OSCC-3 was gift from Mark Lingen (University of Chicago, Chicago). OSCC cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1% penicillin, and 1% streptomycin. Primary human oral keratinocytes (ScienCell, Carlsbad, CA) were maintained in oral keratinocyte medium (OKM), (ScienCell, Carlsbad, CA). $RIP^{-/-}$ mouse embryonic fibroblasts (MEFs) were kindly provided by Philip Leder and Michelle Kellinger (Harvard Medical School, Boston, MA).

### Tissue microarrays

Immunohistochemical analyses were performed to determine the expression of SIRT3 and RIP in human OSCC tissues using OSCC tissue microarrays (OR601 and HN241; US Biomax, Inc., Rockville, MD) and the Histostatin Kit (95-6143; Zymed Laboratories, South San Francisco, CA) according to the manufacturer's instructions. Antibodies to SIRT3 (AP6242a) and RIP (610459) were from ABGENT (San Diego, CA), and Millipore (Billerica, MA), respectively. Staining intensities were graded in a blinded manner as either low (L) or high (H) by a pathologist.

**Transient transfection**

HSC-3 cells at 60–70% confluency were transiently transfected with small interfering RNA (siRNA) (25 or 50 nM) against RIP or with a nontargeting control siRNA (Santa Cruz Biotechnology, Santa Cruz, CA) in serum-free medium that contained Lipofectamine Plus (Invitrogen, Carlsbad, CA). $RIP^{-/-}$ mouse embryonic fibroblasts were transiently transfected with myc-tagged wild-type RIP or control vector as described previously[10]. Transfection efficiency was assessed by Western blot analysis.

**Stable transfection**

HSC-3 and UM-SCC-14A cells were transduced with either RIP-shRNA (sc-44326-V), SIRT3-shRNA (sc61555-vs) or scrambled-shRNA (sc-108084) (Santa Cruz Biotechnology) lentiviral particles in 0.5 mL of serum-free media, then selected in 10 μg/ml puromycin (sc-108071; Santa Cruz Biotechnology) for an additional 10 days. Surviving cell colonies were picked and propagated before testing for RIP or SIRT3 expression using Western blot analysis.

**Immunoblot analysis**

To evaluate the expression levels of RIP and SIRT3, cells were treated as described above or in figure legends, washed once with phosphate-buffered saline, lysed in RIPA buffer (R0278, Sigma) that contained 1% protease inhibitor cocktail (P8340, Sigma), then kept on ice for 30 minutes. Lysates were adjusted for protein concentration with the BCA protein assay kit (Bio-Rad, Hercules, CA). Western blot analyses was performed with various primary antibodies and horseradish peroxidase-conjugated antirabbit or antimouse IgG antibodies, and blots were developed with the ECL-Plus detection system (Pierce, Rockford, Ill). Antibodies for RIP (610459) and SIRT3 (2627) were from Millipore (Billerica, MA) and Cell Signaling Technology (Beverly, MA), respectively. To demonstrate equal protein loading, membranes were stripped and reprobed with an anti-β-actin antibody (sc-1615; Santa Cruz Biotechnology).

**Anoikis-resistant and control adherent OSCC cells**

Anoikis-resistant oraspheres (UM-SCC-14A and HSC-3) and adherent (UM-SCC-14A and HSC-3) OSCC cells were prepared as previously reported[4]. These OSCC cell lines were selected because they represent the extremes of RIP and SIRT3 expression among OSCC cell lines examined. These OSCC cells were developed by maintaining cells under suspension conditions on poly- HEMA coated plates (7.5 mg/ml in 95% ethanol, Sigma) for 6 days, where they survive anchorage withdrawal by forming multicellular aggregate oraspheres. Adherent control cells were maintained in culture medium as described previously.

**Immunodeficient mouse tumor model**

To examine the effects of anoikis-resistance and adherent OSCC cells *in vivo*, UM-SCC-14A and SIRT3 suppressed UM-SCC-14A cells and controls were grown in anoikis resistant and adherent conditions as described above. Four-week-old athymic nude mice (NCr-nu/nu strain, NCI, Frederick, MD) were anesthetized by intraperitoneal injection with 100 mg/kg ketamine and 10 mg/kg xylazine. We used a murine floor-of-mouth model, which we previously optimized to produce 4-mm to 5-mm tumors, corresponding to a palpable tumor volume of 35-60 $mm^3$, within approximately 2 to 4 weeks after injection of tumor cells[14, 16]. Anoikis-resistant and adherent control UM-SCC-14A cells were prepared to a final concentration of 1.0 x $10^6$ /0.05 ml and injected submucosally into the floor of the mouth as described previously[14]. Six weeks after injection, mice were euthanized, tumor volumes were measured by digital caliper using the formula *a x a x b/2,* where *a* is the smaller dimension. Tumor tissues were then harvested, rinsed in PBS, and fixed overnight in 10% buffered formalin. Tissues were paraffin-embedded, sectioned, and processed for routine histopathological assessment with hematoxylin and eosin staining and for SIRT3 and RIP immunostaining.

**Apoptosis cell death detection by ELISA**

Apoptosis was measured *in vitro* by a DNA-fragmentation enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions (Roche Diagnostics, Indianapolis, IN).

**Statistical Analysis**

In general, values are expressed as means ± SD. Intergroup differences were determined by two-way analysis of variance (ANOVA) and Scheffe's multiple-comparison test. Statistical significance was defined as * $p \leq 0.05$. For tissue microarray analyse, McNemar's test was used to compare the two proportions and are considered significantly different when $P \leq .001$. For the in vivo studies, independent t tests with unequal variances were used. All experiments were repeated at least 3 times.

**Results**

**SIRT3 and RIP are oppositely expressed in oral squamous cell carcinoma *in vivo***

We recently reported that SIRT3 is overexpressed in OSCC *in vivo* and *in vitro* compared to other sirtuins and its stable suppression reduces tumor burden in vivo, implicating SIRT3 as a prosurvival and tumor promoting factor[14]. In addition, we showed that RIP plays a critical role in OSCC cells by regulating anoikis through its shuttling between CD95/Fas death and FAK survival signaling pathways, thus demonstrating that RIP acts as a switch between life and death signals in OSCC cells[10]. Also, sirtuin-3 (SIRT3) is known to regulate cell survival, metabolism, and tumorigenesis. Therefore, we hypothesized that SIRT3 might engage in crosstalk with RIP to regulate anoikis-resistance and tumorigenesis in OSCC cells. To test this hypothesis, we first evaluated the native expression levels of SIRT3 and RIP in serial sections of OSCC tissue microarrays (TMAs). Our data included 28 OSCC tongue samples in which SIRT3 and RIP expression were evaluated and assessed as low (L) or high (H) (Table 1). SIRT3 and RIP staining intensity data from Table 3.1 are illustrated in Figure 3.1B. We already knew that SIRT3

expression levels were elevated in human OSCC[14], however the relative expression level of RIP in these same tissues was not known. Interestingly, we found that SIRT3 and RIP were oppositely expressed in OSCC tissues (Fig. 3.1A). In 86% of the samples where SIRT3 expression was high, 75% of them had low RIP expression (Fig. 3.1B; Table 3.1). Thus 64% (18/28) of the specimens showed an opposite expression pattern for SIRT3 and RIP (Fig. 3.1A and B, Table 3.1; $p \leq 0.05$).

**SIRT3 and RIP are oppositely expressed in oral squamous cell carcinoma *in vitro***

Similarly, when we examined several OSCC cells (HSC-3, OSCC-3, and UM-SCC-14A) for SIRT3 and RIP expression, we again found that SIRT3 expression was opposite to that of RIP expression in these cells (Fig. 3.2A). Primary oral keratinocytes also showed an opposite pattern of SIRT3 and RIP expression, however, these cells generally exhibited higher levels of RIP expression compared to SIRT3 (Fig. 3.2A). These findings further suggested that RIP and SIRT3 participate in a crosstalk mechanism. To test this further, OSCC cell lines (HSC-3 and UM-SCC-14A) were selected for further experiments to determine whether RIP or SIRT3 regulate each other and to examine the hierarchy of this potential crosstalk. These OSCC cell lines were chosen because they represent the extremes of SIRT3 and RIP expression among OSCC cell lines examined. Transient suppression of RIP effectively increased SIRT3 expression levels (Fig. 3.2B). However, stable suppression of SIRT3 failed to alter RIP expression levels (Fig. 3.2C). Furthermore, reconstitution of RIP in RIP null cells down-regulated SIRT3 expression (Fig. 3.2D). This indicated that SIRT3 was responsive to changes in RIP levels but not the contrary, suggesting that RIP is a potential upstream regulator of SIRT3.

**Anoikis-resistant OSCC oraspheres express higher levels of SIRT3 and lower levels of RIP**

To examine the anoikis-resistant phenotype of OSCC cells, an orasphere culture assay was used to study the SIRT3 and RIP expression profile of OSCC cells *in vitro*. We specifically developed anoikis-resistant cells and their counterpart adherent controls (HSC-3 and UM-SCC-14A; as described previously). Our

data show that anoikis-resistant OSCC cells in oraspheres maintain cell survival and exhibit low levels of DNA fragmentation or apoptosis (Fig. 3.3A and B). In contrast, the single cell counterpart of suspension cultures undergoes apoptosis and exhibit high levels of DNA fragmentation (Fig. 3.3A and B). The adherent counterpart control cells, like the cells in oraspheres, also have low levels of DNA fragmentation. Interestingly, however, the oraspheres express higher levels of SIRT3 and lower levels of RIP compared to the adherent cells (Fig. 3.3C), suggesting that OSCC cells escape anoikis-mediated cell death in part by relying on SIRT3 survival signaling and suppression of RIP death signaling pathways.

**Anoikis-resistant orasphere-derived OSCC cells that express higher levels of SIRT3 and lower levels of RIP induce a greater tumor burden**

Furthermore, to investigate the *in vivo* relevance of our *in vitro* findings, we used a murine floor-of-mouth model that mimics human OSCC[14, 16, 17]. Mice were injected with anoikis-resistant orasphere-derived OSCC cells that express higher SIRT3 levels (UM-SCC-14A) or their adherent OSCC cell counterparts that express lower SIRT3 levels (Fig 3.4). Our data show that mice injected with anoikis-resistant orasphere-derived cells exhibit greater tumor burden compared to their adherent counterparts (Fig. 3.4A and Table 3.2). Not only did the mice injected with orasphere-derived cells develop significantly larger tumors (12 fold difference in size; $p \leq 0.027$), but they also developed more tumors (9/10), compared to (2/8) mice injected with adherent control cells (Table 3.2; $p \leq 0.001$) Histologic examination of tumors dissected from mice injected with orasphere-derived cells revealed a highly disorganized histologic pattern of invasive epithelial islands with keratin pearl formation, nuclear pleomorphism, hyperchromatism, and increased nuclear to cytoplasmic ratios compared to their control counterparts (Fig. 3.4B). These "aggressive" tumors also revealed high levels of SIRT3 expression and low levels of RIP expression (Fig. 3.4B). In 80% of the samples where SIRT3 expression was high, 78% of them had low RIP expression (Fig. 3.4C). In contrast, the few tumors that emerged in mice injected with adherent control cells revealed a tendency towards regularly organized histologic pattern of epithelial islands and a

relatively lower level of SIRT3 expression (data not shown). These data support our *in vitro* findings and those of others[6, 7] indicating that anoikis-resistant cells induce a greater tumor burden and a more aggressive phenotype in OSCC. In addition, these cells seem to rely, at least in part, on SIRT3 upregulation and RIP downregulation to maintain their survival and aggressive phenotype. Thus, high SIRT3 and low RIP expression may impart resistance to anoikis-mediated cell death.

**SIRT3 upregulation and RIP downregulation regulate the fate of OSCC cells in anoikis conditions**

To further validate the importance of SIRT3 and RIP in regulating the fate of OSCC cells under suspension/anoikis conditions, stable cell clones of SIRT3-shRNA (Clone2) and scrambled-shRNA (Clone 1) were examined under adherent and suspension conditions. Our data show that under suspension conditions, cells expressing SIRT3-shRNA can no longer form oraspheres, maintain their survival, or escape anoikis, unlike cells expressing scrambled-shRNA (Fig. 3.5A), confirming the role of SIRT3 in anoikis resistance. In addition, cells in suspension conditions stably expressing SIRT3-shRNA (oraspheres and single cells) showed increased levels of RIP expression and higher levels of DNA fragmentation compared to cells expressing SIRT3-shRNA and grown in adherent conditions or cells expressing scrambled-shRNA and grown in adherent or suspension conditions (Fig. 3.5B and C). Furthermore, stable suppression of SIRT3 inhibits anoikis resistance and reduces tumor incidence *in vivo* (Fig. 3.5D). The higher RIP expression present in the cells transduced with SIRT3-shRNA in suspension conditions likely represents the single cell fraction present in this mixed culture system. Furthermore, RIP overexpression promotes anoikis[10] and RIP suppression inhibits DNA fragmentation (Fig. 3.6). These data show that SIRT3 and RIP are at opposite ends of anoikis resistance in OSCC cells, and SIRT3 is required for escaping anoikis and for acquisition of an anoikis-resistant phenotype. Based on these findings, we propose a model for SIRT3's role in regulating survival and anoikis resistance (Fig. 3.7).

**Discussion**

Cancers that are prone to metastases possess an anoikis-resistant phenotype, thereby acquire a more aggressive behavior, resistance to treatment, and contributing to poor survival rates. This has been the case for several cancers, such as prostate, hepatic, and oral cancer[6, 18, 19]. Thus, understanding the molecular mechanisms underlying an anoikis-resistant phenotype will help identify new potential therapeutic targets to treat aggressive cancer.

Our previous report that RIP can shuttle between survival and death signaling pathways under anoikis conditions in oral cancer[10], demonstrates the important role of RIP in controlling the fate of OSCC cells under anoikis conditions. In addition, our recent finding that OSCC cells rely on SIRT3 to maintain their survival and aggressive behavior also suggests an important role for SIRT3 in OSCC tumorigenesis. Specifically, we found that SIRT3 downregulation decreased OSCC cell proliferation and survival, enhanced the sensitivity of OSCC radio- and chemo-resistant cells to both radio- and chemotherapeutic treatments, and reduced tumor burden *in vivo*[14]. Here, we show for the first time a link between anoikis-resistance and SIRT3 in oral cancer and its potential cross-talk with RIP, thus highlighting one important new mechanism by which SIRT3 can modulate OSCC progression.

Our data show that SIRT3 and RIP are oppositely expressed in OSCC and that OSCC cells escape anoikis by forming multicellular aggregates or oraspheres to maintain their survival compared to single cells, which undergo anoikis-mediated cell death. Interestingly, OSCC cells seem to rely, at least in part, on altering their SIRT3 and RIP levels to escape anoikis. Furthermore, OSCC cells stably expressing downregulated levels of SIRT3 failed to form oraspheres, and thus experienced significantly more anoikis, suggesting a critical role for SIRT3 in regulating anoikis-resistance in OSCC cells. In agreement with our findings, lung adenocarcinoma cells form spheroids or aggregates that express E-cadherin and p-Src to maintain their survival under suspension conditions. Thus, Src was found to be an essential regulator in the development of anoikis-resistance in lung adenocarcinomas[20]. Also, mammosphere

cultures of breast cancer cells from pleural effusions are tumorigenic and induce tumors in SCID mice[5]. Furthermore, a recent report demonstrate that ovarian cancer spheroids use integrin- and talin-dependent activation of myosin and traction forces to promote displacement of mesothelial cells from underneath a tumor cell spheroid[22]. In summary, multiple studies support the concept that spheroid formation promotes cancer cell survival and tumorigenesis.

Limited studies have examined the role of anoikis-resistance in OSCC progression and aggressive behavior using *in vivo* models. One study used a tongue oral cancer mouse model to show that anoikis-resistant OSCC cells are more aggressive than their anoikis-sensitive counterparts, and mice injected with these cells have shorter survival rates (17 days versus 30 days) compared to mice injected with anoikis-sensitive OSCC cells[6]. In our current study, we used a different *in vivo* model, a murine floor-of-mouth model that mimics human OSCC[16, 17], and showed that anoikis-resistant OSCC cells, which express increased levels SIRT3 and decreased levels of RIP, exhibit greater tumor burden compared to their adherent OSCC counterparts. These data further support our previous *in vivo* findings, whereby OSCC cells with stably suppressed levels of SIRT3 injected in the same murine floor-of-mouth model exhibited reduced tumor burden compared to controls[14]. Thus, our current data support that SIRT3 and RIP are oppositely expressed in OSCC anoikis-resistant cells and this enables these cells to escape anoikis and take on a more aggressive phenotype. Since this short 6-week floor-of-mouth model is not optimal for examination of oral metastases, other animal models of metastases are needed to examine this process specifically in future studies.

RIP can localize to the cytoplasm or the mitochondria. In the mitochondria, RIP inhibits ADP/ATP exchange via modulating adenine nucleotide translocate (ANT). This modulation results in the loss of ANT and cyclophilin-D interactions, reduction in ATP levels, and induction of cell death[21], and implicates RIP in mitochondrial-mediated cell death. In addition, although there is debate about the subcellular localization of SIRT3, most reports support a mitochondrial localization for SIRT3[22-30]. Our

data show that SIRT3 and RIP are oppositely expressed in OSCC and they regulate anoikis-resistance in OSCC cells. Since SIRT3, a mitochondrial sirtuin, can localize to the mitochondrial matrix, and RIP can be localized in the cytoplasm[31, 32], this suggests that these two molecules may interact indirectly via other molecules. However, additional studies indicate that RIP is also identified in the mitochondria, and therefore can potentially interact directly with SIRT3[21, 33]. Furthermore, some evidence suggests that RIP is critical for regulating ROS mechanisms[34, 35] and it is well known that SIRT3 is functionally also important in ROS regulation[15, 36, 37]. Therefore, RIP and SIRT3 may be functionally related via ROS regulation pathways in the context of anoikis resistance. However, whether this regulation is mediated via direct or indirect interactions between SIRT3 and RIP is an area that is currently under investigation by our group.

There is also a debate in the literature regarding the role of SIRT3 in cancer[15]. Although, some reports support a prosurvival role for SIRT3 in cancer[14, 15, 38-41], others support a tumor suppressor role for SIRT3[42-44]. Our previous and current studies demonstrate that SIRT3 plays a prosurvival role in oral cancer, that SIRT3 assists in chemo- and radioresistance, and that by promoting anoikis-resistance, SIRT3 mediates a more aggressive tumorigenic phenotype.

In summary, our studies reveal for the first time a novel role for SIRT3 as a modulator of anoikis-resistance in oral cancer via potential cross-talk with RIP. This work further enriches our understanding of SIRT3's role in the regulation of oral cancer tumorigenesis and implicates SIRT3 as new potential therapeutic target to treat oral cancer.

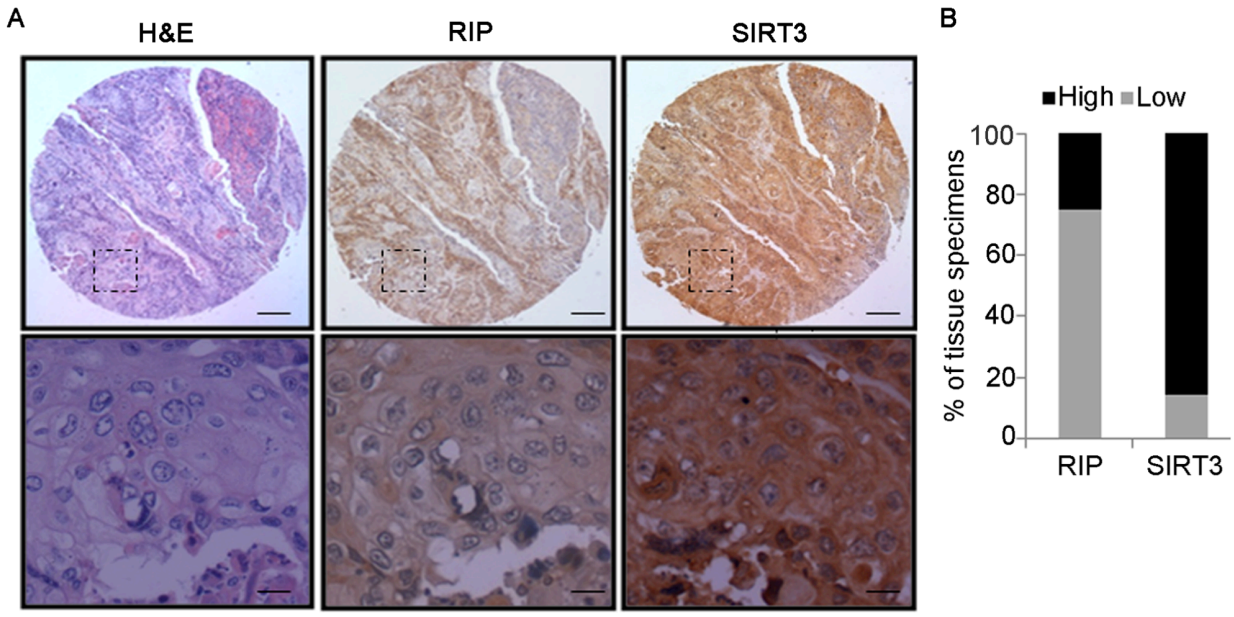


**Figure 3.1: RIP expression shows an opposite relationship to SIRT3 expression in oral squamous cell carcinoma (OSCC). (**A) RIP and SIRT3 expression in OSCC TMA specimens are illustrated. Scale bars, 200 μm for low-magnification photographs (top) and 50 μm for high-magnification photomicrographs (bottom). (B) Percentage of OSCC tissue specimens expressing RIP and SIRT3 in tongue serial sections, determined by immunohistochemical staining. Staining intensity was graded as High or Low. McNemar's test was used to compare the two proportions and are significantly different, $P \leq .001$.

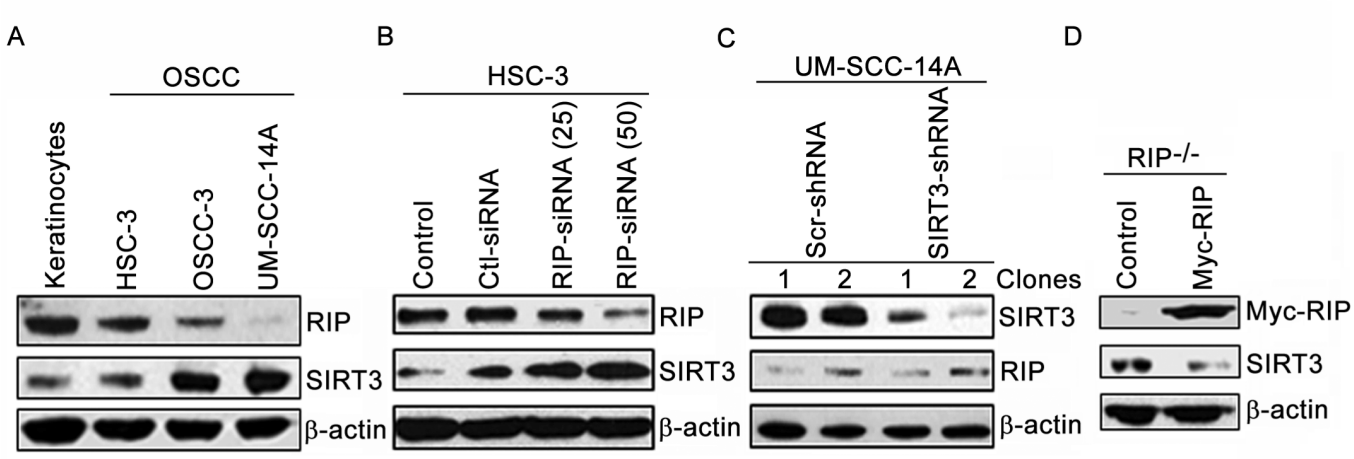


**Figure 3.2: RIP may be an upstream negative regulator of SIRT3. (**A) Immunoblots show RIP and SIRT3 levels in normal human keratinocytes and oral squamous cell carcinoma cells (OSCC) plated for one day. β-actin served as loading control. (B) Immunoblots show RIP and SIRT3 levels after transfection with control siRNA or RIP siRNA (25 or 50 nM) in HSC-3 cells for 30 h (C) Immunoblots show SIRT3 and RIP levels after stable SIRT3 suppression using lentiviral particles (scrambled controls or SIRT3-shRNA) in UM-SCC-14A cells. (D). Immunoblots show RIP and SIRT3 levels after transfection with wild-type myc-RIP in RIP null cells ($RIP^{-/-}$) for 30 h.

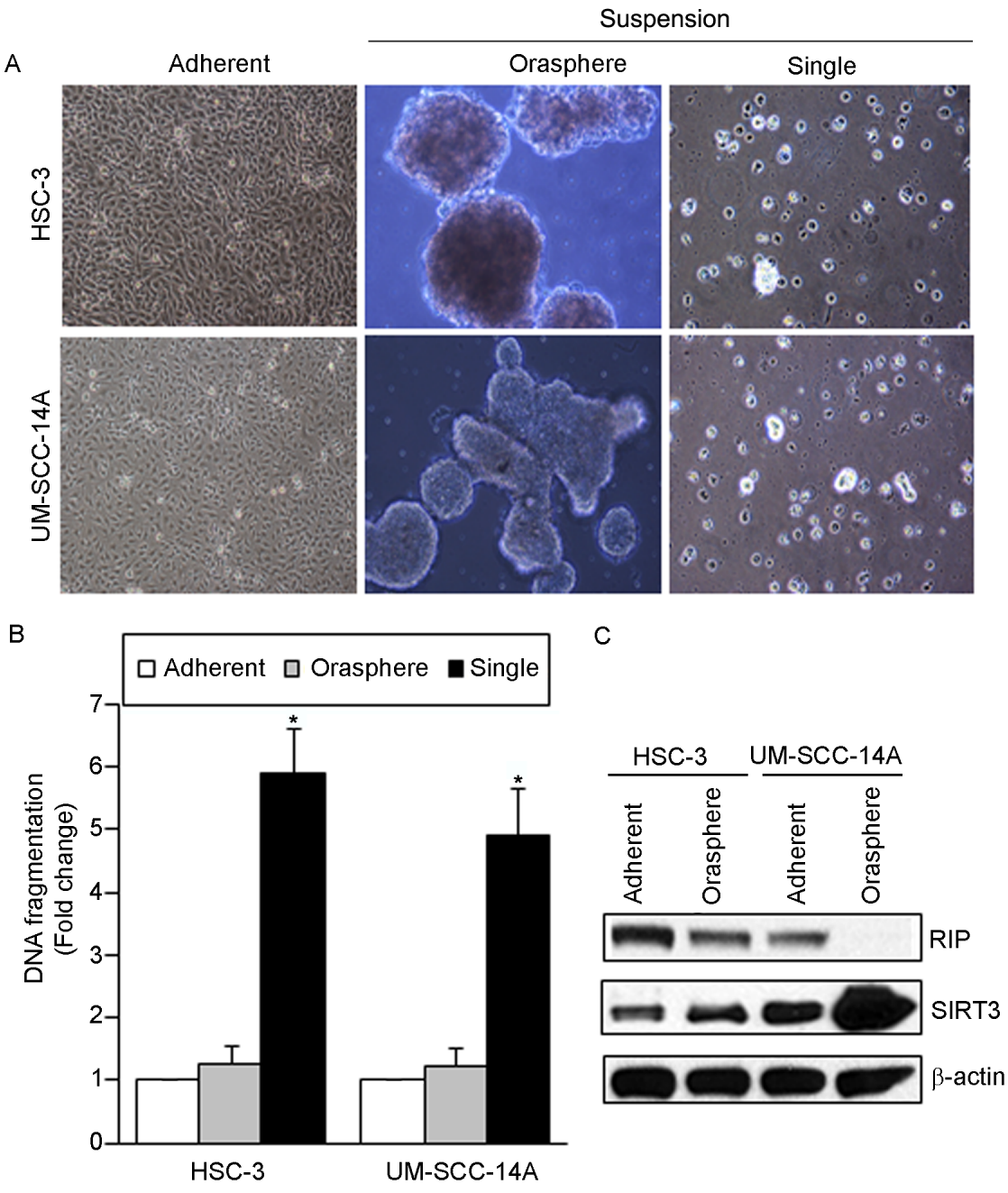


**Figure 3.3: As OSCC cells become anoikis resistant their SIRT3 expression increases as their RIP expression decreases. (**A**)** Phase contrast images of OSCC cells (HSC-3 and UM-SCC-14A) cultured under adherent or suspension conditions for 6 days and orasphere and single cells were separated for analysis. (B) Fold change of DNA fragmentation in adherent, orasphere and single cells. (C) Immunoblots showing RIP and SIRT3 expression in adherent and orasphere.

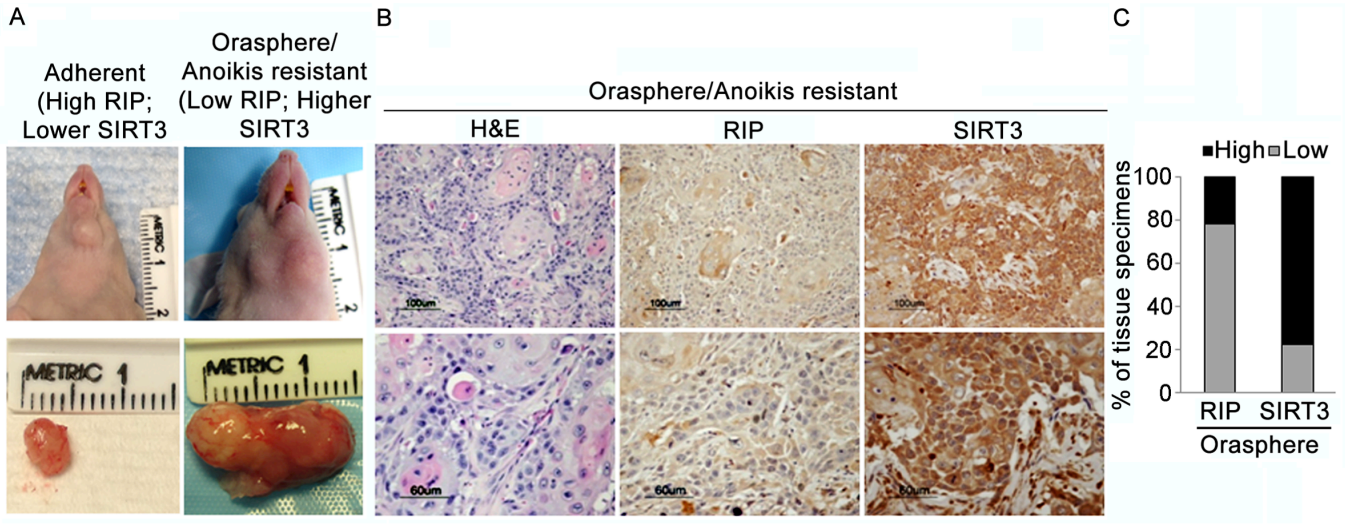


**Figure 3.4: Anoikis resistant OSCC cells induce greater tumor burden in mice.** (A) Images of tumor-bearing mice 6 weeks after injection with adherent or orasphere/anoikis resistant UM-SCC-14A cells. Top panels show superficial tumors and lower panels show dissected tumor. (B) Representative orasphere derived tumor section stained with H&E (left) and immnostained with antibodies for RIP (middle) and SIRT3. (C) Percentage of tissue specimens expressing RIP and SIRT3 in orasphere derived tumor sections, determined by immunohistochemical staining. Staining intensity was graded as High or Low. McNemar's test was used to compare the two proportions and are significantly different, $P \leq .001$.

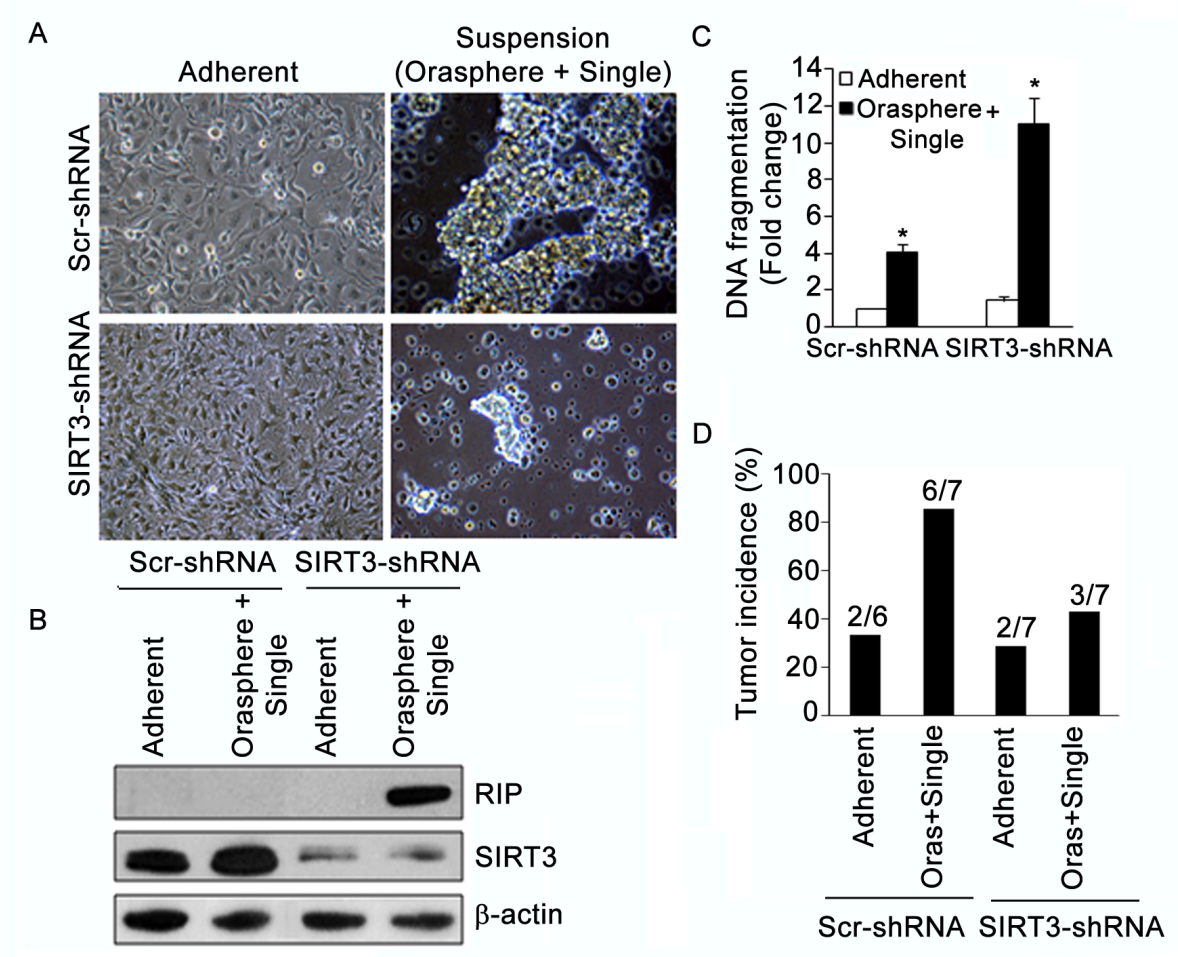


**Figure 3.5: SIRT3 suppression blocks orasphere formation, inhibits anoikis resistance, and reduces tumor incidence *in vivo*.** (A) Phase contrast images of UM-SCC-14A cells transduced with scrambled shRNA (Scr-shRNA) or SIRT3 shRNA (viral transduction and puromycin selection for 10 days) then cultured under adherent or suspension (oraspheres plus single cells) conditions for 6 days. (B) Immunoblots show RIP and SIRT3 levels in scrambled controls or SIRT3 suppressed UM-SCC-14A cells cultured under adherent or suspension (oraspheres/anoikis-resistant) conditions for 6 days. (C) Fold change in DNA fragmentation in adherent or suspension (orasphers plus single cells) conditions for 6 days. (D) Percentage of tumor incidence in nude mice injected with SIRT3 suppressed UM-SCC-14A cells (SIRT3-shRNA) or scrambled controls (Scr-shRNA) from adherent or suspension (oraspheres plus single cells) conditions after 6 weeks. Tumor incidence is shown relative to the number of animals in each group.

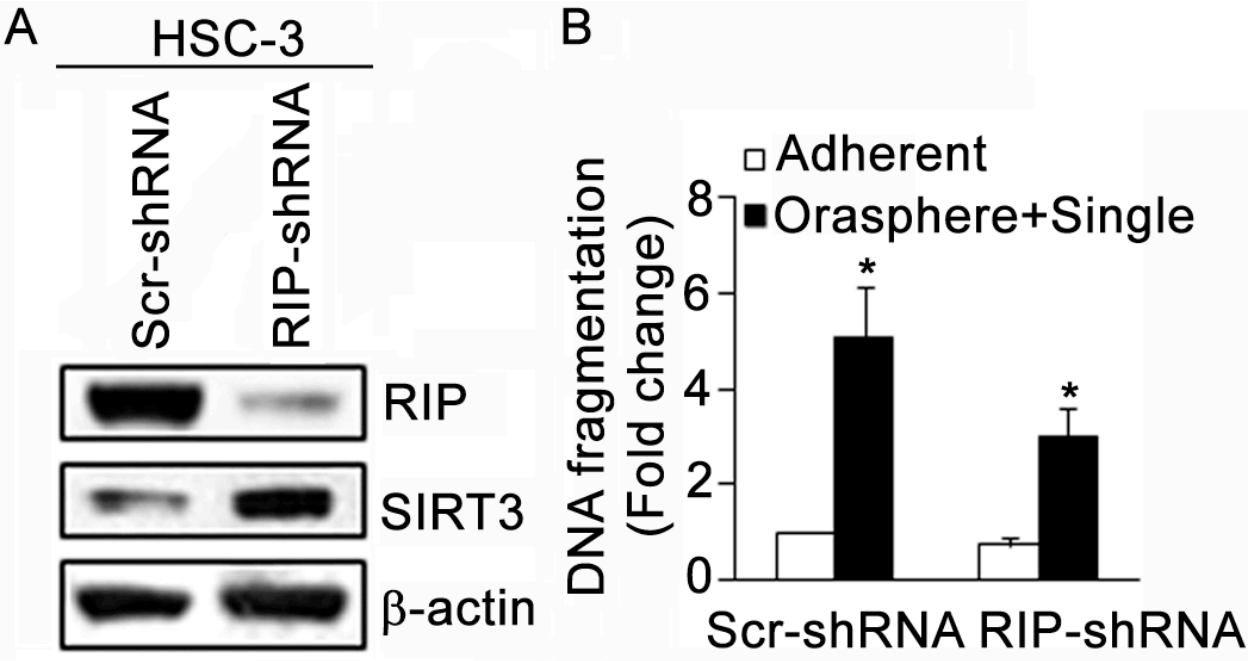


**Figure 3.6: RIP suppression inhibits DNA fragmentation.** (A) Immunoblots show RIP and SIRT3 levels after stable RIP suppression using lentiviral particles (scrambled controls or RIP-shRNA). (B) Fold change in DNA fragmentation in adherent or suspension (oraspheres plus single cells) conditions for 6 days.

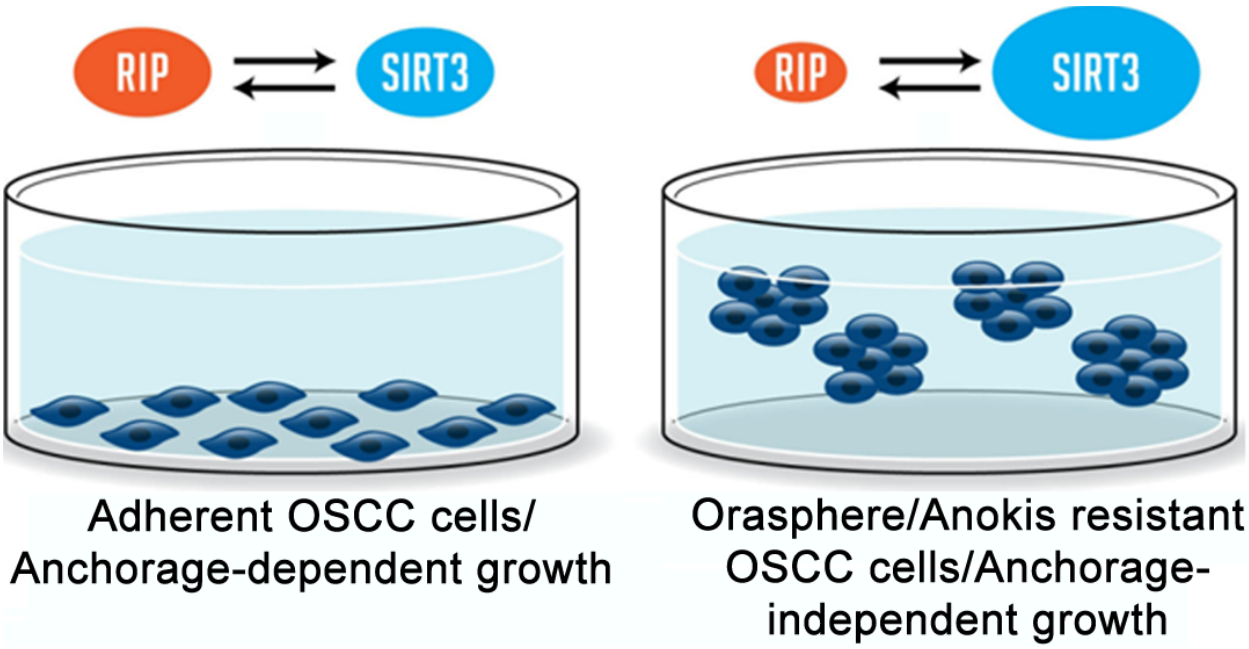


**Figure 3.7: Working model of anoikis resistance.** Anoikis-resistant cells form multicellular aggregates (oraspheres) that express higher SIRT3 levels as their RIP expression decreases thereby promoting their survival and more aggressive phenotype in OSCC development and progression.

Table 3.1. The expression profile of RIP and SIRT3 from 28 different tongue TMAs samples

| Sample number | Section number | Sex | Age | RIP | SIRT3 |
|---|---|---|---|---|---|
| 1 | A1 | M | 57 | L | H |
| 2 | A6 | F | 35 | H | H |
| 3 | A7 | M | 78 | L | H |
| 4 | A9 | F | 39 | L | L |
| 5 | A10 | F | 46 | L | H |
| 6 | B3 | F | 57 | L | H |
| 7 | B4 | F | 36 | L | H |
| 8 | B8 | F | 47 | H | H |
| 9 | B9 | F | 63 | L | L |
| 10 | B10 | M | 56 | L | L |
| 11 | C2 | F | 55 | L | H |
| 12 | C3 | M | 76 | L | H |
| 13 | C4 | F | 50 | L | H |
| 14 | C6 | M | 55 | H | H |
| 15 | C9 | M | 60 | L | H |
| 16 | D1 | M | 64 | L | H |
| 17 | D2 | F | 52 | L | H |
| 18 | D3 | F | 50 | L | H |
| 19 | D4 | F | 46 | L | H |
| 20 | D5 | F | 45 | L | H |
| 21 | D6 | M | 35 | L | H |
| 22 | D7 | F | 46 | H | H |
| 23 | D8 | F | 48 | H | L |
| 24 | E3 | M | 60 | H | H |
| 25 | E4 | M | 37 | L | H |
| 26 | E6 | M | 60 | L | H |
| 27 | E8 | M | 60 | H | H |
| 28 | E10 | M | 73 | L | H |

Staining intensity was graded as Low (L) or High (H)

Table 3.2. Tumor volumes for mice injected with UM-SCC-14A

| | Adherent | Orasphere/ Anoikis resistant |
|---|---|---|
| Animal | Tumor Volume ($mm^3$) | Tumor Volume ($mm^3$) |
| 1 | 31.20 | 518.9 |
| 2 | 19.64 | 444.36 |
| 3 | No tumor | 22.86 |
| 4 | No tumor | 864 |
| 5 | No tumor | 243.3 |
| 6 | No tumor | 252 |
| 7 | No tumor | 215.66 |
| 8 | No tumor | 138.16 |
| 9 | _ | 83.49 |
| 10 | _ | No tumor |
| Mean Volume | 25.42* | 311.84* |

Statistical analysis: Independent t-test with unequal variances $p \leq 0.027$

**References**


1. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100(1):57-70.

2. Freedman VH, Shin SI. Cellular tumorigenicity in nude mice: correlation with cell growth in semi-solid medium. *Cell* 1974;3(4):355-9.

3. Shin SI, Freedman VH, Risser R, Pollack R. Tumorigenicity of virus-transformed cells in nude mice is correlated specifically with anchorage independent growth in vitro. *Proc Natl Acad Sci U S A* 1975;72(11):4435-9.

4. Kantak SS, Kramer RH. E-cadherin regulates anchorage-independent growth and survival in oral squamous cell carcinoma cells. *J Biol Chem* 1998;273(27):16953-61.

5. Grimshaw MJ, Cooper L, Papazisis K, et al. Mammosphere culture of metastatic breast cancer cells enriches for tumorigenic breast cancer cells. *Breast Cancer Res* 2008;10(3):R52.

6. Swan EA, Jasser SA, Holsinger FC, Doan D, Bucana C, Myers JN. Acquisition of anoikis resistance is a critical step in the progression of oral tongue cancer. *Oral Oncol* 2003;39(7):648-55.

7. Kupferman ME, Patel V, Sriuranpong V, et al. Molecular analysis of anoikis resistance in oral cavity squamous cell carcinoma. *Oral Oncol* 2007;43(5):440-54.

8. Bunek J, Kamarajan P, Kapila Y. Anoikis mediators in oral squamous cell carcinoma. *Oral Dis*;17(4):355-61.

9. Bsoul SA, Huber MA, Terezhalmy GT. Squamous cell carcinoma of the oral tissues: a comprehensive review for oral healthcare providers. *J Contemp Dent Pract* 2005;6(4):1-16.

10. Kamarajan P, Bunek J, Lin Y, Nunez G, Kapila YL. Receptor-interacting protein shuttles between cell death and survival signaling pathways. *Mol Biol Cell*;21(3):481-8.

11. Michan S, Sinclair D. Sirtuins in mammals: insights into their biological function. *Biochem J* 2007;404(1):1-13.

12. Verdin E, Hirschey MD, Finley LW, Haigis MC. Sirtuin regulation of mitochondria: energy production, apoptosis, and signaling. *Trends Biochem Sci*;35(12):669-75.

13. Huang JY, Hirschey MD, Shimazu T, Ho L, Verdin E. Mitochondrial sirtuins. *Biochim Biophys Acta*;1804(8):1645-51.

14. Alhazzazi TY, Kamarajan P, Joo N, et al. Sirtuin-3 (SIRT3), a novel potential therapeutic target for oral cancer. *Cancer*.

15. Alhazzazi TY, Kamarajan P, Verdin E, Kapila YL. SIRT3 and cancer: Tumor promoter or suppressor? *Biochim Biophys Acta*;1816(1):80-88.

16. Henson B, Li F, Coatney DD, et al. An orthotopic floor-of-mouth model for locoregional growth and spread of human squamous cell carcinoma. *J Oral Pathol Med* 2007;36(6):363-70.

17. Wolter KG, Wang SJ, Henson BS, et al. (-)-gossypol inhibits growth and promotes apoptosis of human head and neck squamous cell carcinoma in vivo. *Neoplasia* 2006;8(3):163-72.

18. Cao Y, Fu YL, Yu M, et al. Human augmenter of liver regeneration is important for hepatoma cell viability and resistance to radiation-induced oxidative stress. *Free Radic Biol Med* 2009;47(7):1057-66.

19. Sakamoto S, McCann RO, Dhir R, Kyprianou N. Talin1 promotes tumor invasion and metastasis via focal adhesion signaling and anoikis resistance. *Cancer Res* 2010;70(5):1885-95.

20. Sakuma Y, Takeuchi T, Nakamura Y, et al. Lung adenocarcinoma cells floating in lymphatic vessels resist anoikis by expressing phosphorylated Src. *J Pathol* 2010;220(5):574-85.

21. Temkin V, Huang Q, Liu H, Osada H, Pope RM. Inhibition of ADP/ATP exchange in receptor-interacting protein-mediated necrosis. *Mol Cell Biol* 2006;26(6):2215-25.

22. Bao J, Lu Z, Joseph JJ, et al. Characterization of the murine SIRT3 mitochondrial localization sequence and comparison of mitochondrial enrichment and deacetylase activity of long and short SIRT3 isoforms. *J Cell Biochem*;110(1):238-47.

23. Cooper HM, Huang JY, Verdin E, Spelbrink JN. A new splice variant of the mouse SIRT3 gene encodes the mitochondrial precursor protein. *Plos One* 2009;4(3):e4986.

24. Cooper HM, Spelbrink JN. The human SIRT3 protein deacetylase is exclusively mitochondrial. *Biochem J* 2008;411(2):279-85.

25. Jin L, Galonek H, Israelian K, et al. Biochemical characterization, localization, and tissue distribution of the longer form of mouse SIRT3. *Protein Sci* 2009;18(3):514-25.

26. Lombard DB, Alt FW, Cheng HL, et al. Mammalian Sir2 homolog SIRT3 regulates global mitochondrial lysine acetylation. *Mol Cell Biol* 2007;27(24):8807-14.

27. Michishita E, Park JY, Burneskis JM, Barrett JC, Horikawa I. Evolutionarily conserved and nonconserved cellular localizations and functions of human SIRT proteins. *Mol Biol Cell* 2005;16(10):4623-35.

28. Onyango P, Celic I, McCaffery JM, Boeke JD, Feinberg AP. SIRT3, a human SIR2 homologue, is an NAD-dependent deacetylase localized to mitochondria. *Proc Natl Acad Sci U S A* 2002;99(21):13653-8.

29. Schwer B, North BJ, Frye RA, Ott M, Verdin E. The human silent information regulator (Sir)2 homologue hSIRT3 is a mitochondrial nicotinamide adenine dinucleotide-dependent deacetylase. *J Cell Biol* 2002;158(4):647-57.

30. Shi T, Wang F, Stieren E, Tong Q. SIRT3, a mitochondrial sirtuin deacetylase, regulates mitochondrial function and thermogenesis in brown adipocytes. *J Biol Chem* 2005;280(14):13560-7.

31. Stanger BZ, Leder P, Lee TH, Kim E, Seed B. RIP: a novel protein containing a death domain that interacts with Fas/APO-1 (CD95) in yeast and causes cell death. *Cell* 1995;81(4):513-23.

32. Kelliher MA, Grimm S, Ishida Y, Kuo F, Stanger BZ, Leder P. The death domain kinase RIP mediates the TNF-induced NF-kappaB signal. *Immunity* 1998;8(3):297-303.

33. Kasof GM, Prosser JC, Liu D, Lorenzi MV, Gomes BC. The RIP-like kinase, RIP3, induces apoptosis and NF-kappaB nuclear translocation and localizes to mitochondria. *FEBS Lett* 2000;473(3):285-91.

34. Shen HM, Lin Y, Choksi S, et al. Essential roles of receptor-interacting protein and TRAF2 in oxidative stress-induced cell death. *Mol Cell Biol* 2004;24(13):5914-22.

35. Lin Y, Choksi S, Shen HM, et al. Tumor necrosis factor-induced nonapoptotic cell death requires receptor-interacting protein-mediated cellular reactive oxygen species accumulation. *J Biol Chem* 2004;279(11):10822-8.

36. Ahn BH, Kim HS, Song S, et al. A role for the mitochondrial deacetylase Sirt3 in regulating energy homeostasis. *Proc Natl Acad Sci U S A* 2008;105(38):14447-52.

37. Kong X, Wang R, Xue Y, et al. Sirtuin 3, a new target of PGC-1alpha, plays an important role in the suppression of ROS and mitochondrial biogenesis. *Plos One*;5(7):e11707.

38. Ashraf N, Zino S, Macintyre A, et al. Altered sirtuin expression is associated with node-positive breast cancer. *Br J Cancer* 2006;95(8):1056-61.

39. Li S, Banck M, Mujtaba S, Zhou MM, Sugrue MM, Walsh MJ. p53-induced growth arrest is regulated by the mitochondrial SirT3 deacetylase. *Plos One*;5(5):e10486.

40. Sundaresan NR, Gupta M, Kim G, Rajamohan SB, Isbatan A, Gupta MP. Sirt3 blocks the cardiac hypertrophic response by augmenting Foxo3a-dependent antioxidant defense mechanisms in mice. *J Clin Invest* 2009;119(9):2758-71.

41. Yang H, Yang T, Baur JA, et al. Nutrient-sensitive mitochondrial NAD+ levels dictate cell survival. *Cell* 2007;130(6):1095-107.

42. Allison SJ, Milner J. SIRT3 is pro-apoptotic and participates in distinct basal apoptotic pathways. *Cell Cycle* 2007;6(21):2669-77.

43. Kim HS, Patel K, Muldoon-Jacobs K, et al. SIRT3 is a mitochondria-localized tumor suppressor required for maintenance of mitochondrial integrity and metabolism during stress. *Cancer Cell*;17(1):41-52.

44. Marfe G, Tafani M, Indelicato M, et al. Kaempferol induces apoptosis in two different cell lines via Akt inactivation, Bax and SIRT3 activation, and mitochondrial dysfunction. *J Cell Biochem* 2009;106(4):643-50.

# CHAPTER IV

## A NOVEL SIRTUIN-3 (SIRT3) INHIBITOR, LC-0296, INHIBITS CELL PROLIFERATION AND SURVIVAL, AND PROMOTES APOPTOSIS IN HEAD AND NECK CANCER CELLS


## ABSTRACT

The poor survival rate of head and neck squamous cell carcinoma (HNSCC), which stands at approximately 50%, underscores the need to explore new areas of research and develop new therapeutic drugs and approaches that can help improve the survival rate of head and neck cancer patients. Sirtuins (SIRT1-7) have emerged as important modulators of different tumorigenic processes, thus implicating sirtuin modifiers as new potential therapeutic approaches to treat cancer. In this report, we show for the fist time a novel role for a SIRT3 inhibitor, LC-0296, in inhibiting cell survival and proliferation, and promoting apoptosis in HNSCC cells, but not in normal human oral keratinocytes. This inhibitory effect is mediated, at least in part, via modulating reactive oxygen species (ROS) levels. In addition, LC-0296 works synergistically to increase the sensitivity of HNSCC cells to both radiation and cisplatin treatments. In summary, the development of novel SIRT3 inhibitors, such as LC-0296, might enable the development of new targeted therapies to treat and improve the survival rate of head and neck cancer patients.


### Introduction

Head and neck squamous cell carcinoma of (HNSCC) is the type of cancer that originates from the oral and nasal cavities, sinuses, lips, salivary glands, throat, or larynx. Oral squamous cell carcinoma (OSCC) represents the majority of HNSCC and it is the eighth most common cancer worldwide [1]. In some countries, including India and other south-central Asian countries, oral cancer is among the most commonly occurring cancers. Although other cancers such as

breast, prostate and colon cancers are more prevalent than oral cancer, the 5-year survival rate of this disease is poor, averaging 50% [1]. Despite the advancements in therapeutic approaches to treat this devastating disease, oral cancer still holds one of the worse survival rates compared to other cancer types worldwide. In the U.S., one person is estimated to die every hour from oral cancer [1], whereas in Canada, three people die from this disease hourly [2]. This underscores the urgent need to explore new areas of research and develop new therapeutic drugs and approaches that can help improve the survival rate of head and neck cancer patients.

Sirtuins (SIRT1-7) have emerged as important modulators of different tumorigenic processes. Sirtuins control cancer cell proliferation and survival, cell cycle progression, apoptosis, angiogenesis, and metabolism [3,4,5,6]. Therefore, sirtuins have been implicated as novel potential therapeutic targets to treat cancer [7]. However, the role of several sirtuins, specifically SIRT1 and SIRT3, in cancer tumorigenesis has been controversial [8,9,10]. Thus, expanding the study of sirtuins in this new area of research will help advance the field and help us to better understand the mechanisms by which sirtuins can regulate different cancer processes.

We were the first to demonstrate a novel role for SIRT3 in oral cancer tumorigenesis *in vitro* and *in vivo* [11]. We previously reported that of all the seven sirtuin family members, SIRT3 is overexpressed in OSCC compared to normal oral tissues, and SIRT3 downregulation enhances the sensitivity of OSCC cells to both radiation and chemotherapeutic drugs. In addition, SIRT3 downregulation inhibits OSCC cell growth and proliferation *in vitro*, and reduces tumor burden *in vivo* [11]. Furthermore, we found that SIRT3 and receptor interacting protein (RIP), a proapoptotic protein, are oppositely expressed in OSCC human tumor specimens. In addition, OSCC cells escape anoikis, apoptotic cell death triggered by loss of extracellular matrix (ECM) contacts, by forming multicellular aggregates or oraspheres to maintain their survival, unlike single cells, which undergo anoikis-mediated cell death (Y. Kapila's group, unpublished data under revision). Thus, OSCC oraspheres become anoikis-resistant, a condition defined by a

higher SIRT3 and low RIP expression. These anoikis-resistant OSCC cells also induce an increased tumor burden and incidence in mice unlike their adherent OSCC cell counterparts. Furthermore, stable suppression of SIRT3 inhibits anoikis-resistance and reduces tumor incidence (Y. Kapila's group, unpublished data under revision). These findings suggest that SIRT3 may be a new potential therapeutic target to treat head and neck cancer patients.

In this regard, we believe that discovering new drugs that specifically target SIRT3 could enhance the treatment of HNSCC cancer and this would be of a great interest for potentially improving the survival rate of head and neck cancer patients. In this study, we report for the first time the use of a novel SIRT3 chemical inhibitor, LC-0296, that retards OSCC cell proliferation and survival, and promotes apoptosis through the modulation of reactive oxygen species (ROS). In addition, inhibiting SIRT3 enzymatic activity with this inhibitor increases the sensitivity of HNSCC cells to radiation and chemotherapeutic treatments. To our knowledge, our report is the first to show a promising new specific SIRT3 chemical inhibitor that can form the basis for future therapeutics for head and neck cancer patients.

## Materials and Methods

**Brief description of chemical synthesis and reaction conditions.** The synthesis of compound LC-0296 (**6**) was straightforward and is depicted in Figure 4.S1. Commercially available 4-nitro-1*H*-indole (**1**) was alkylated to give compound **3**, whose nitro group was reduced to the corresponding amine in compound **4** in excellent yields. The methyl ester group in compound **4** was converted into a primary amide with methanolic ammonia. The resulting compound **5** was coupled with L-glutamate derived Z-Glu-OMe in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and 1-hydroxybenzotriazole (HOBt) to yield LC-0296 in good yields (Figure 4.1). More detailed information about the chemical synthesis can be found in supplementary methods (Figure 4.S1 and Methods 4.S1).

**Biochemical *in vitro* assays.** The biochemical assays against human SIRT1-3 were performed by the Reaction Biology Incorporation (RBC) (Malvern, PA, USA, http://www.reactionbiology.com). Full length human SIRT1 (GenBank Accession No. NM_012238, MW = 82 kDa), full length human SIRT2 (GenBank Accession No. NM_012237, MW = 43 kDa), and catalytically active human SIRT3 (GenBank Accession No. NM_012239, amino acids 102-399, MW = 32.7 kDa) were expressed in *E. coli* and purified. All assays were performed using a fluorogenic 7-amino-4-methylcoumarin (AMC)-labeled substrate (RHKK(Ac)-AMC), which is based on residues 379–382 of p53. The assays were performed in a buffer of 50 mM Tris-HCl (pH 8.0), 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$ and 1 mg/ml BSA, which was added before use. The testing involved a two-step reaction. First, 50 μM AMC-labeled substrate with an acetylated lysine side chain was incubated with 91 nM SIRT1 for 2 hours at 30 ºC to produce the deacetylated substrate. The concentrations of SIRT2 and SIRT3 were 233 and 917 nM, respectively. Second, the deacetylated substrate was digested by a mixture of developer to release AMC that was detected at 360/460 Ex/Em. The intensity of fluorescence was proportional to the amount of the deacetylated substrate. Each compound was dissolved in DMSO, sequentially diluted, and used for testing. $IC_{50}$ values were calculated from the resulting sigmoidal dose-response curves. Suramin was used as a reference compound for SIRT1 as well as SIRT2, and nicotinamide for SIRT3.

**Cell lines and culture.** Human HNSCC cell lines, UM-SCC-1 and UM-SCC-17B, originated from the floor of the mouth and larynx, respectively, and were from Tom Carey (University of Michigan). HNSCC cells were maintained in DMEM (Gibco) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin. Primary normal human oral keratinocytes (Cat# 2610, ScienCell) were maintained in human oral keratinocytes medium (#2611, ScienCell).

**Cell viability and colony formation assays.** To determine the effect of the SIRT3 inhibitor, LC-0296, on cell viability we used the QUANT Cell Proliferation Assay Kit according to manufacturer's instructions (Invitrogen), under the conditions described in figure legends. For colony formation assays, HNSCC cells and normal human oral keratinocytes were treated and cultured for one week. Colonies were fixed with methanol, and then stained with 0.5% crystal violet. Colonies containing greater than 50 cells were counted.

**Apoptosis cell death detection assay (ELISA).** To measure apoptosis *in vitro*, a DNA fragmentation enzyme-linked immunosorbent assay (ELISA) was used according to manufacturer's instructions (Roche Diagnostics, Indianapolis, IN, USA).

**Combination Index (CI).** To determine synergistic or additive effects of the drug combinations, a CI was used following the procedure developed by Fischel et al. [12], with the equation adapted from the method developed previously by Chou and Talalay [13]:

$$R = \frac{\text{Survival (LC-0296 + IR or CDDP)}}{\text{Survival (LC-0296 alone) X (IR or CDDP alone)}}$$

According to Fischel et al. [12], if:

1. $R < 0.8$, then the association is considered to be synergistic;

2. $0.8 < R < 1.2$, then the association is considered to be additive;

3. $R > 1.2$, then the association is considered to be antagonistic.

**Immunoblot analysis.** Western blotting was performed as previously described [11]. The SIRT3 antibody (#2627) was from Cell Signaling. To demonstrate equal protein loading, membranes were stripped and reprobed with an anti-β-actin antibody (sc-1615, Santa Cruz Biotechnology).

**Determination of SIRT3 deacetylation activity in HNSCC cells.** To determine the effect of LC-0296 on SIRT3 deacetylation activity *in vivo*, HNSCC cells were either control (DMSO) or LC-0296 (50 μM) for 18 h. Cells were then lysed and samples were adjusted for protein concentration with the BCA protein assay kit (Bio Rad). Samples were then assayed for SIRT3 deacetylation activity using the fluorogenic SIRT3 Assay Kit (#50083, BPS Bioscience) according to manufacturer's instructions.

**Reactive Oxygen Species (ROS) detection assay:** To measure the intracellular ROS levels under the conditions described in the figure legends, the fluorogenic marker for ROS, carboxy-H2DCFDA, was used according to manufacturer's instructions (Invitrogen).

**Statistical analysis.** Values were expressed as means ± SD. Comparisons between groups were determined by one-way analysis of variance (ANOVA) followed by Tukey-HSD multiple-comparison test. Statistical significance was defined as $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$. All experiments were repeated three times and at least in triplicates when applicable.

## Results

### Defining the SIRT3 inhibitor, LC-0296, its chemical structure and its enzymatic selectivity *in vitro*

The chemical structure and the schematic chemical reaction of LC-0296 are shown in Figure 4.1A and Figure 4.S1, respectively. The SIRT3 inhibitor, LC-0296, possesses a selective inhibitory effect, about ~20 and 10 fold more inhibition on SIRT3 enzymatic activity compared to SIRT1 and SIRT2, as shown in Table 4.1.

**SIRT3 inhibitor, LC-0296, inhibits HNSCC cell survival without affecting normal human oral keratinocytes**

To assess the effect of the SIRT3 inhibitor, LC-0296, on HNSCC cell survival, we first performed dose- and time dependent experiments using a wide range of doses (.001, .01, .1, 1, and 10 μM, in 24, 48, and 72 h), and compared those to normal human oral keratinocytes (Figure 4.S1). Since we did not see significant inhibitory effects on HNSCC cell survival with these doses, we tested higher doses of LC-0296. At doses in the μM range, LC-0296 showed significant inhibitory and dose-dependent effects on HNSCC cell viability (UM-SCC-1 and UM-SCC-17B) without affecting normal human oral keratinocytes (Figure 4.1B). A representative image of HNSCC cells (75 μM) treated with LC-0296 is presented in Figure 4.1C. This further demonstrates the absence of significant quantitative or morphological changes in the normal human oral keratinocytes treated with LC-0296 (Figure 4.1B and C).

**SIRT3 inhibitor, LC-0296, inhibits cell growth and proliferation and promotes apoptosis in HNSCC cells**

To further evaluate the effect of the SIRT3 inhibitor, LC-0296, on HNSCC cell growth and proliferation, colony formation assays were performed using different doses. Our data demonstrate that in addition to inhibiting cell viability, the SIRT3 inhibitor, LC-0296, also blocked HNSCC cell colony formation in a dose-dependent manner (Fig. 4.2A). Notably, the 75 μM dose had an even greater effect on colony formation ability than on cell viability as shown in Figure 1B. This may be explained by the fact that single cell colony growth assays are devoid of growth signals from neighboring cells and cell-cell contact, thereby promoting a less robust survival environment. In addition, LC-0296 not only retarded HNSCC cell growth and proliferation, but it also induced apoptosis in a dose-dependent manner (Figure 4.2B and C). Importantly, LC-0296, did not exert significant effects on cell growth and proliferation or apoptosis on normal human keratinocytes (Figure 4.2D-F).

**SIRT3 inhibitor, LC-0296, enhances the sensitivity of HNSCC cells to both radiation and chemotherapeutic drugs**

Thus far our data indicate that the SIRT3 inhibitor, LC-0296, can be used as a single agent to inhibit HNSCC cell growth and survival, and promote apoptosis-mediated cell death. However, we further asked whether LC-0296 could function as an effective adjuvant treatment for those cases that do not respond well to either radiation or chemotherapeutic approaches. Both UM-SCC-1 and UM-SCC-17B cells are very aggressive HNSCC cell lines, which are highly resistant to radiation [14,15], and the former is also resistant to cisplatin (CDDP) (data not shown). Interestingly, LC-0296 worked synergistically to enhance the sensitivity of HNSCC cell lines to both treatments compared to untreated controls or cells treated with radiation or CDDP alone (Figure 4.3A and B). The synergistic effect was assessed using a combination index (CI). It is noteworthy that the synergistic effect was obtained using the 50 μM dose, which is below the $IC_{50}$ of LC-0296 in both HNSCC cell lines (Figure 4.3A and B).

**SIRT3 inhibitor, LC-0296, inhibits SIRT3 deacetylation activity in HSNCC cells**

Previously, we showed that downregulation of SIRT3 levels in OSCC cells significantly limits OSCC cell survival and aggressiveness *in vitro* and *in vivo* [11]. Although, our novel SIRT3 chemical inhibitor can selectively inhibit SIRT3 enzymatic activity *in vitro* (Table 4.1), it was not known whether LC-0296, functions by inhibiting SIRT3 deacetylation activity *in vivo* within a cellular context. Therefore, we first assayed for SIRT3 deacetylation activity using cell lysates of the two HNSCC cell lines treated with the SIRT3 inhibitor (LC-0296, 50 μM), and compared that to untreated controls (DMSO vehicle control, 50 μM). In addition, we performed Western blot analyses to assess the effect of LC-0296 on SIRT3 protein levels *in vivo*. Our results show that LC-0296, inhibits SIRT3 deacetylation activity *in vivo* without affecting SIRT3 protein levels (Figure 4.4A and B).

**SIRT3 inhibitor, LC-0296, retards cell survival and enhances apoptosis via modulating ROS levels in HNSCC cells**

Several studies support that normal cells have lower ROS levels compared to cancer cells [16,17], and it is well known that SIRT3 has a key regulatory role in controlling ROS levels in the mitochondria [6,18]. Therefore, we hypothesized that the SIRT3 inhibitor, LC-0296, might be affecting SIRT3 deacetylation activity, by modulating ROS levels in these cells, and thereby affecting their survival and promoting apoptosis. Thus, since ROS levels are higher in cancer cells compared to normal cells, this may explain why LC-0296, was more effective on HNSCC cells compared to normal human oral keratinocytes. To test this hypothesis, we evaluated ROS levels in HNSCC cells compared to normal human oral keratinocytes. In agreement with previous studies in other cancer cells [16,17], ROS levels in HNSCC cells were significantly higher than those in normal human oral keratinocytes (Figure 4.5A). Next, we measured ROS levels in HNSCC cells following LC-0296 treatment. Interestingly, we did find a significant increase in ROS levels in the LC-0296 treated HNSCC cells compared to untreated vehicle controls (Figure 4.5B). In addition, to further confirm that LC-0296 works in HNSCC cells by modulating ROS levels, we next used N-Acetyl-Cysteine (NAC), a scavenger for ROS in this context. Indeed, addition of NAC abrogated the effect of LC-0296 on HNSCC cells, thus decreasing ROS levels, increasing cell viability and decreasing apoptosis (Figure 4.5B-D). Importantly, the modulation of ROS levels in HNSCC cells by LC-0296, was associated with retarded cell survival and enhanced apoptosis (Figure 4.5C and D). These data show that the SIRT3 inhibitor, LC-0296, possesses a specific inhibitory effect on HNSCC cell viability, and enhances apoptosis, at least in part, by modulating ROS levels.

**Discussion**

The poor survival rate for head and neck cancer, urgently summons the development of new areas of research that might identify new strategies and approaches for drug development or the

discovery of new targets or markers, that could aid in the early diagnosis and treatment of patients with head and neck cancer.

Sirtuins (SIRT1-7) has been extensively investigated for just over a decade. This field of study is an exciting area that seems to hold great promise toward enhancing our understanding of and aiding in the development of treatments for age-related diseases such as diabetes, neurodegenerative disorders, heart disease, and cancer [4,19]. However, since sirtuin biology is still in an early stage of development, there are controversial viewpoints as to the significance of several sirtuins in the area of cancer biology. SIRT1 and SIRT3 seem to be at the focus of this controversy [8,9,10,20]. We have devoted our research over the last several years to investigating the role of sirtuins in head and neck cancer. Interestingly, we found that of all the known sirtuins (SIRT1-7), SIRT3 is specifically overexpressed in oral cancer *in vitro* and *in vivo* [11]. This overexpression was associated with increased aggressiveness and tumorigenesis *in vivo*. Furthermore, SIRT3 downregulation resulted in reduced tumor burden *in vivo,* and an enhanced sensitivity to both radiation and chemotherapeutic drugs *in vitro* [11]. In addition, our group recently found a role for sirtuins, specifically SIRT3, in promoting anoikis resistance. We found that SIRT3 expression levels increased in OSCC cells as they developed an anoikis-resistant phenotype compared to their adherent counterparts. This higher SIRT3 expression was associated with lower RIP expression levels, and increased tumor burden and incidence *in vivo*. Furthermore, SIRT3 and RIP were oppositely expressed in OSCC tissues. Importantly, stable suppression of SIRT3 blocked the anoikis resistant phenotype *in vitro* and tumor burden *in vivo*. Thus, SIRT3 plays a role in anoikis-resistance that likely contributes to tumorigenesis and aggressiveness of OSCC (Y. Kapila's group, unpublished data under revision).

In this study, we report on a novel SIRT3 inhibitor, LC-0296, that has shown an enhanced selectivity toward inhibiting SIRT3 deacetylation activity. Importantly, LC-0296, shows specificity toward retarding HNSCC cell survival and enhancing apoptosis, without affecting

normal human oral keratinocytes. This inhibitor functioned as both a single agent and in combination with either radiation or cisplatin (CDDP) treatment to block HNSCC cell viability, especially in cell lines that were derived from patients that have shown resistance to these treatments.

The role of ROS in carcinogenesis/tumorigenesis is well documented in the literature [21,22]. ROS is responsible for normal cell transformation, thus promoting tumorigenesis [21,22]. However, it is important to keep in mind that normal cells still need low levels of ROS for physiologic functions. Thus, the balance between ROS production and antioxidants seems to be a key factor in controlling normal cellular processes or abnormal cellular transformation [23,24]. SIRT3 seems to be a key regulatory switch in the mitochondria, keeping mitochondrial integrity and protecting it from increased ROS levels, thus preventing ROS-mediated cell transformation to cancer via deacetylating and activating mitochondrial antioxidants such as MnSOD and SOD2 [25,26,27]. However, normal cells in general posses lower ROS levels compared to cancer cells [16,17], Thus, it seems that after cellular transformation, cancer cells undergo an adaptation to increased ROS levels that signifies part of their abnormal genomic dysregulation. Interestingly, the balance of ROS levels seems to be a sensor for cellular survival and proliferation, and responsible for the increased aggressive phenotype and resistance to conventional cancer treatments [22,23,24,28]. Therefore, because SIRT3 is overexpressed in HNSCC cells [11], for a reason that is yet unknown, and as part of the abnormal genomic dysregulation in these cells, we believe that SIRT3 may be responsible for controlling the balance of ROS levels in these cancer cells. SIRT3 may be keeping ROS levels at a threshold that promotes cancer cell survival, and thereby promoting a more aggressive phenotype that resists conventional cancer treatments. Given these ideas, we investigated whether LC-0296 works via ROS modulation in HNSCC cells. Interestingly, because ROS levels are higher in HNSCC cells compared to normal keratinocytes, LC-0296 seems to alter the balance of ROS levels in cancer cells toward retarding cell survival,

and enhancing apoptosis. This may also explain, at least in part, the increased sensitivity of IR and CDDP resistant HNSCC cells to this drug, especially, when used in combination treatments. Although, several studies have shown that the antioxidant, NAC, may function as a chemopreventive agent, and it inhibits tumorigenesis in several cancer types including, brain, melanoma, and prostate cancers [29,30,31], our data show that in a short pretreatment period, NAC alone, did not have a significant effect of HNSCC cell viability and apoptosis. However, this pretreatment was sufficient to retard the inhibitory effect of LC-0296 on these cells via the reduction of ROS levels. Taking advantage of increased ROS levels in cancer cells is one strategy for achieving more potent and selective effects of chemotherapeutic drugs. There are several ROS based chemical inhibitors that have been developed and tested in clinical trials with promising results [32,33].

To our knowledge, there are no published reports on clinical trials using class-III histone deacetylase inhibitors (HDAC) of sirtuins to treat cancer. Our current report represents a proof of principle wherein treatment with SIRT3 inhibitors may be advantageous for overcoming HNSCC aggressiveness and drug resistant phenotypes. Our novel SIRT3 inhibitor, LC-0296, is the first generation of this type of drug, Our group is currently working on developing more potent and suitable versions of this drug that may be more applicable for testing within an *in vivo* setting and for potential future clinical trials.

In summary, the development of novel SIRT3 inhibitors, such as LC-0296 may open new avenues for discovery and development of targeted therapies for HNSCC by taking advantage of the increased ROS levels in HNSCC. This may result in better treatment outcomes that are associated with fewer side effects, a better quality of life, and improved survival rates for head and neck cancer patients.

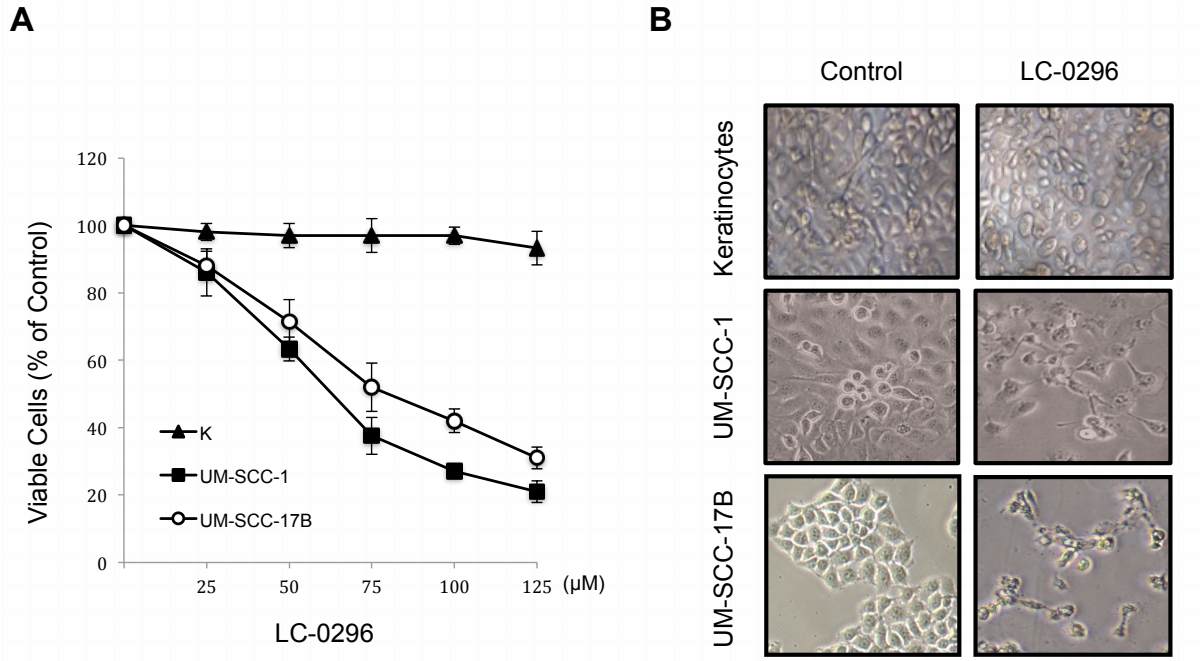


**Figure 4.1: SIRT3 inhibitor, LC-0296, inhibits OSCC cell survival without affecting normal human oral keratinocytes.** (A) The chemical structure of the SIRT3 inhibitor, LC-0296. (B) HNSCC cells (UM-SCC-1 and UM-SCC-17B) and normal human oral keratinocytes (k) were seeded in 96 well plate at 5 x$10^3$ cells/well, then treated with LC-0296, as indicated for 24 h. Cell viability was determined by the QUANT Cell Proliferation Assay Kit (Invitrogen). (C) Phase contrast images showing the morphology of HNSCC cells (UM-SCC-1 and UM-SCC-17B) and normal human oral keratinocytes, after treated with control (DMSO) or LC-0296 (75 μM) for 24 h. *p*-value, $^{***}p \leq 0.001$ LC-0296 vs control (50, 75, 100, and 125 μM).

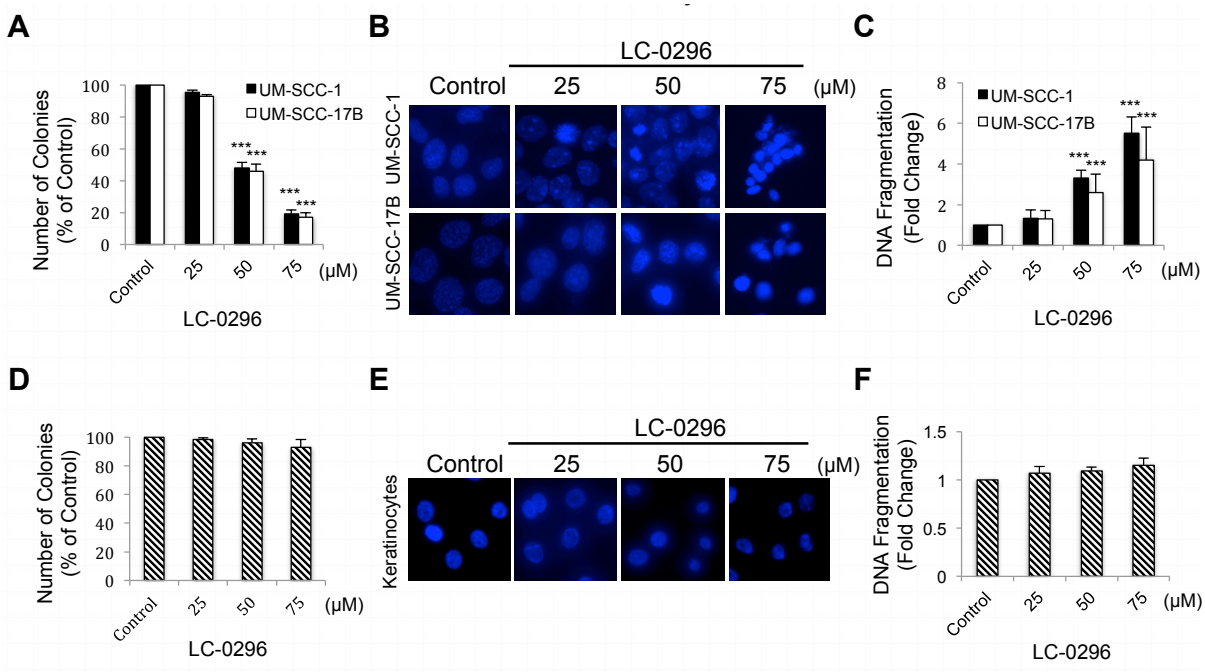


**Figure 4.2: SIRT3 inhibitor, LC-0296, inhibits cell growth and proliferation and promotes apoptosis in HNSCC cells.** (A) Histograms representing the quantification of colony forming assay of HNSCC cells (UM-SCC-1 and UM-SCC-17B). Cells were seeded in 6-well plate at 250 cells/well, treated with LC-0296, as indicated, then cultured for one week. Colonies were fixed with methanol, then stained with crystal violet. Number of colonies were presented as the percentage of colonies obtained relative to controls. (B) Representative images of HNSCC cells (UM-SCC-1 and UM-SCC-17B) stained with DAPI after treated with Control (DMSO) or LC-0296 as indicated for 24 h. Culture media was collected and centrifuged to collect floating cells, which were added back to their respective wells. (C) Fold change of DNA fragmentation in HNSCC cells after treated with control (DMSO) or LC-0296 as indicated for 24 h. (D) Histograms representing the quantification of colony forming assay, (E) representative images DAPI stained cells, (F) fold change of DNA fragmentation, in normal human oral keratinocytes (K) after treated with Control (DMSO) or LC-0296 as indicated for 24 h. $^{***}p \leq 0.001$.

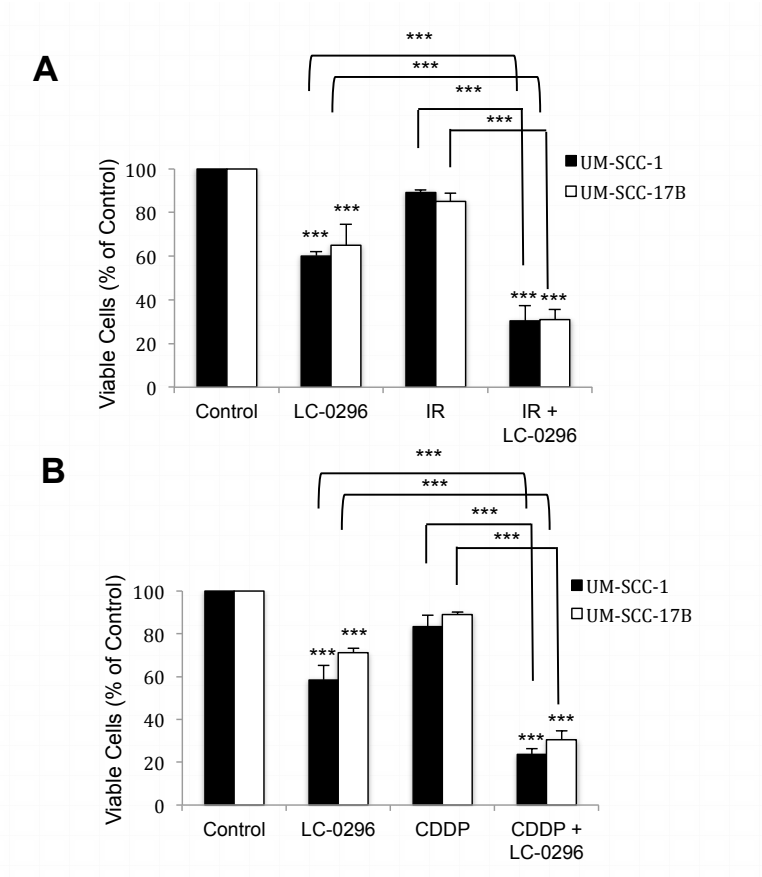


**Figure 4.3: SIRT3 inhibitor, LC-0296, enhances the sensitivity of HNSCC cells to both radiation and chemotherapeutic drugs.** (A) HNSCC cells were treated with LC-0296 (50 μM), with or without ionizing radiation (IR, 2.5 Gy) or cisplatin (CDDP, 20 μM) (B) for 24 h, then cytotoxicity was determined by the QUANT Cell Proliferation Assay Kit. Control (treatment with DMSO vehicle, 50 μM for 24 h). $^{***}p \leq 0.001$.

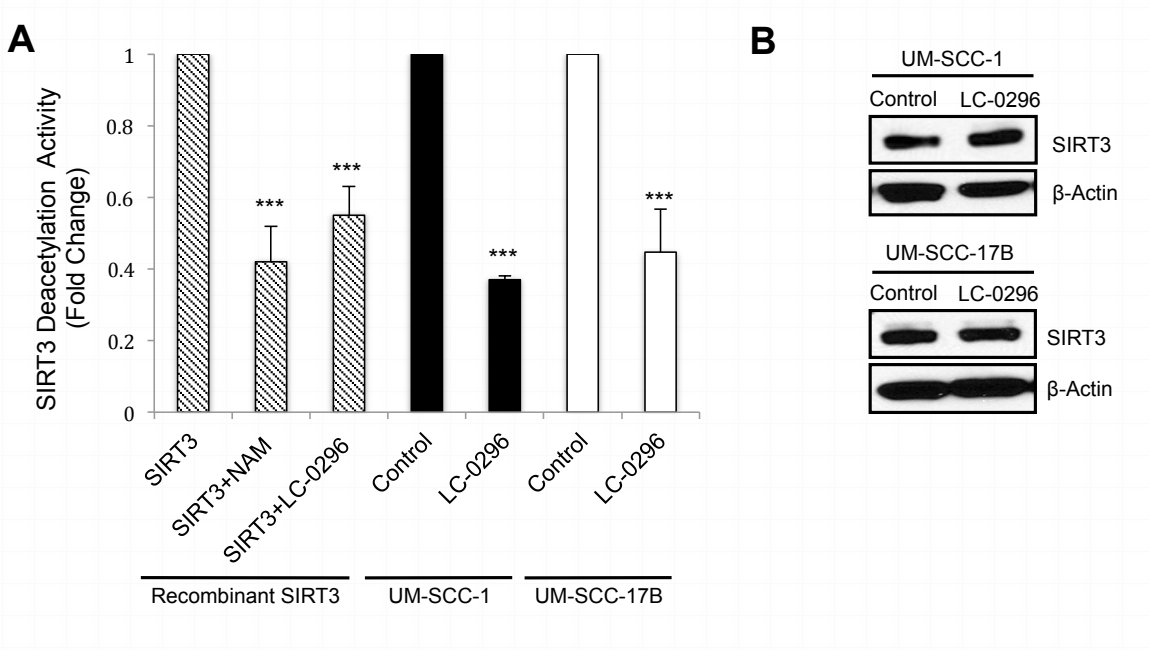


**Figure 4.4: SIRT3 inhibitor, LC-0296, inhibits SIRT3 deacetylation activity in HSNCC cells.** (A) HNSCC cells (UM-SCC-1 and UM-SCC-17B) were treated with control (DMSO) or LC-0296 (50 μM) for 24 h and lysates were assayed for SIRT3 deacetylation using fluorogenic SIRT3 assay kit. Recombinant SIRT3 and NAM, were used as positive controls as per manufacturer's instructions. $^{***}p \leq 0.001$. (B) Immunoblots showing SIRT3 expression levels in HNSCC cells after treated with LC-0296 as indicated. β-Actin served as loading control.

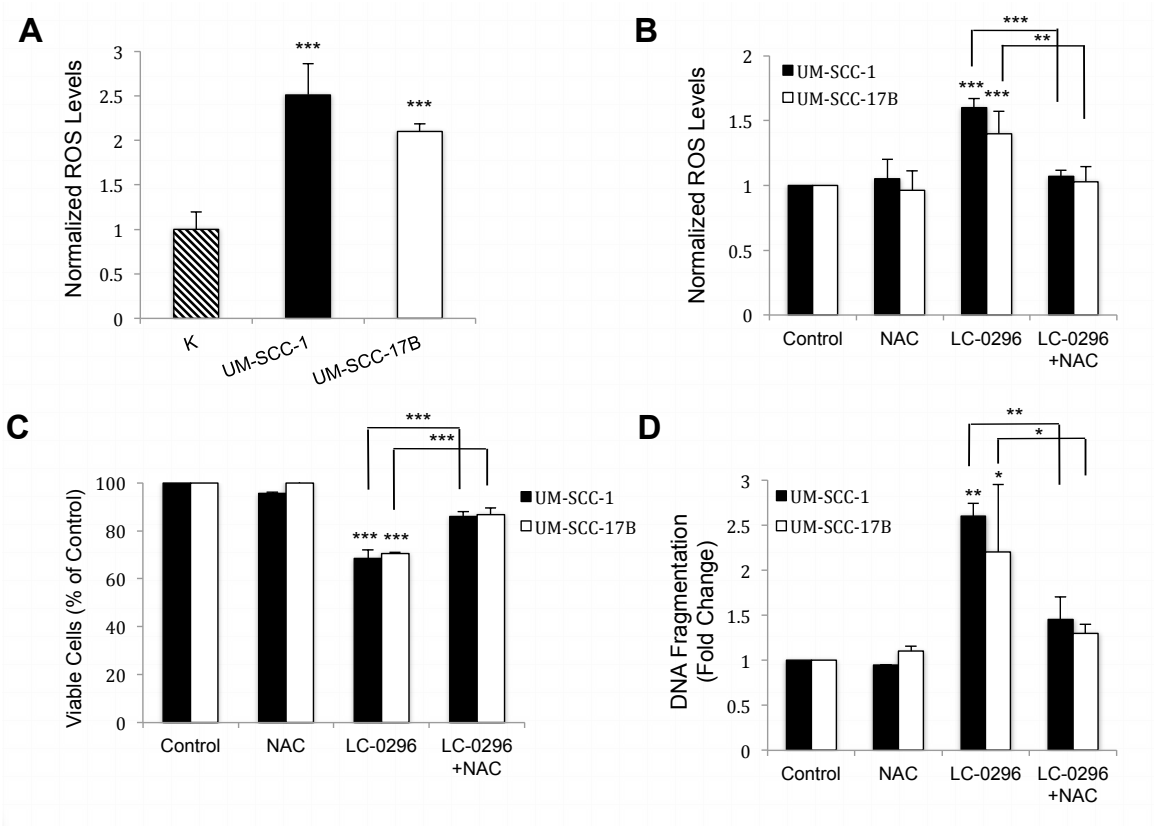


**Figure 4.5: SIRT3 inhibitor, LC-0296, retards cell survival and enhances apoptosis via modulating ROS levels in HNSCC cells.** (A) Normal human oral keratinocytes (K), and HNSCC cells (UM-SCC-1 and UM-SCC17B) were seeded in 96 well-plate at 5 x $10^3$ cells/ well, then ROS levels were measured after 24 h, using the fluorogenic marker carboxy-H2DCFDA. (B) HNSCC cells were pretreated with N-Acetyl-Cysteine (NAC, 20 mM), a scavenger for ROS, or control (DMSO) for 2 h, then the pretreatments were removed, and cells were subsequently treated with either control (DMSO) or LC-0296 (50 μM) for 10 h, and finally stained with carboxy-H2DCFDA for ROS assays. (C) HNSCC cells were treated as in panel B for 12 h, and then cell viability was determined by the QUANT Cell Proliferation Assay. (D) HNSCC cells were treated as in panel B for 12 h, and then apoptosis was determined using DNA fragmentation ELISA. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$.

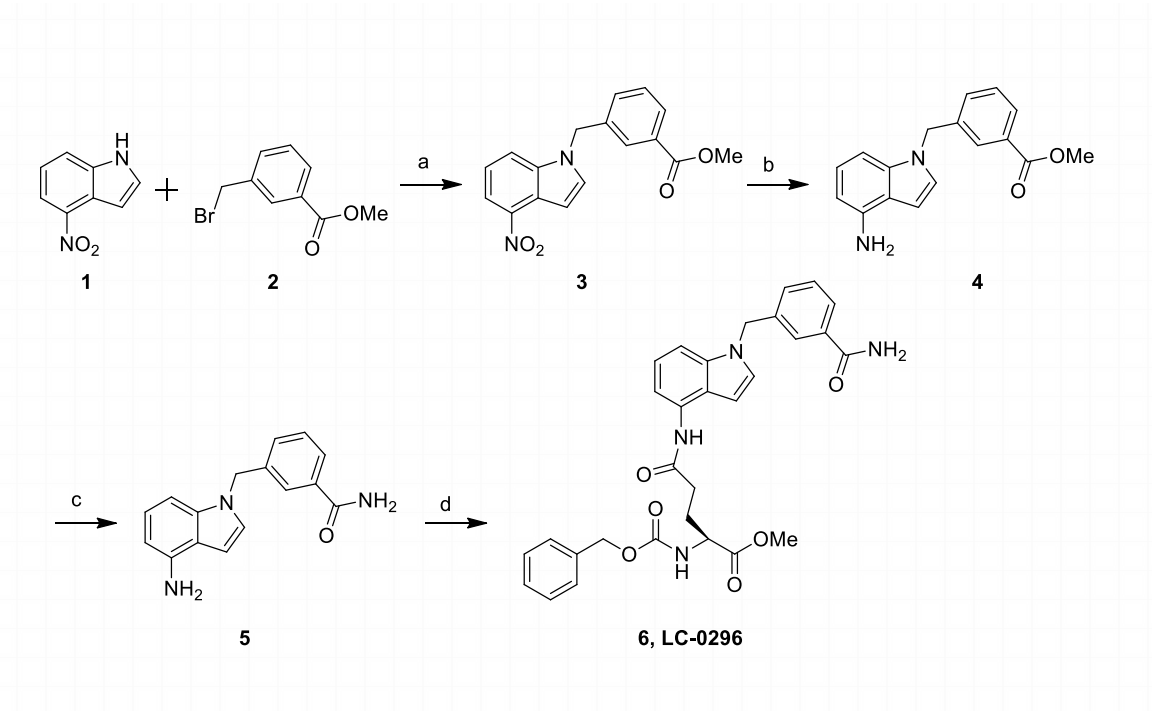


**Figure 4.S1: Schematic chemical synthesis of SIRT3 Inhibitor (LC-0296)**

Commercially available 4-nitro-1*H*-indole (**1**) was alkylated to give compound **3**, whose nitro group was reduced to the corresponding amine in compound **4** in excellent yields. The methyl ester group in compound **4** was converted into a primary amide with methanolic ammonia. The resulting compound **5** was coupled with L-glutamate derived Z-Glu-OMe in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 1-hydroxybenzotriazole (HOBt) to yield LC-0296 in good yields. Reaction conditions (a) NaH, DMF, yield 68%; (b) $NiCl_2 \cdot 6H_2O$, $NaBH_4$, MeOH, yield 89%; (c) methanolic $NH_3$, $CaCl_2$, 70 ºC, yield 85%; (d) Z-Glu-OMe, EDC, HOBt, NMM, $CH_2Cl_2$, yield 60%.

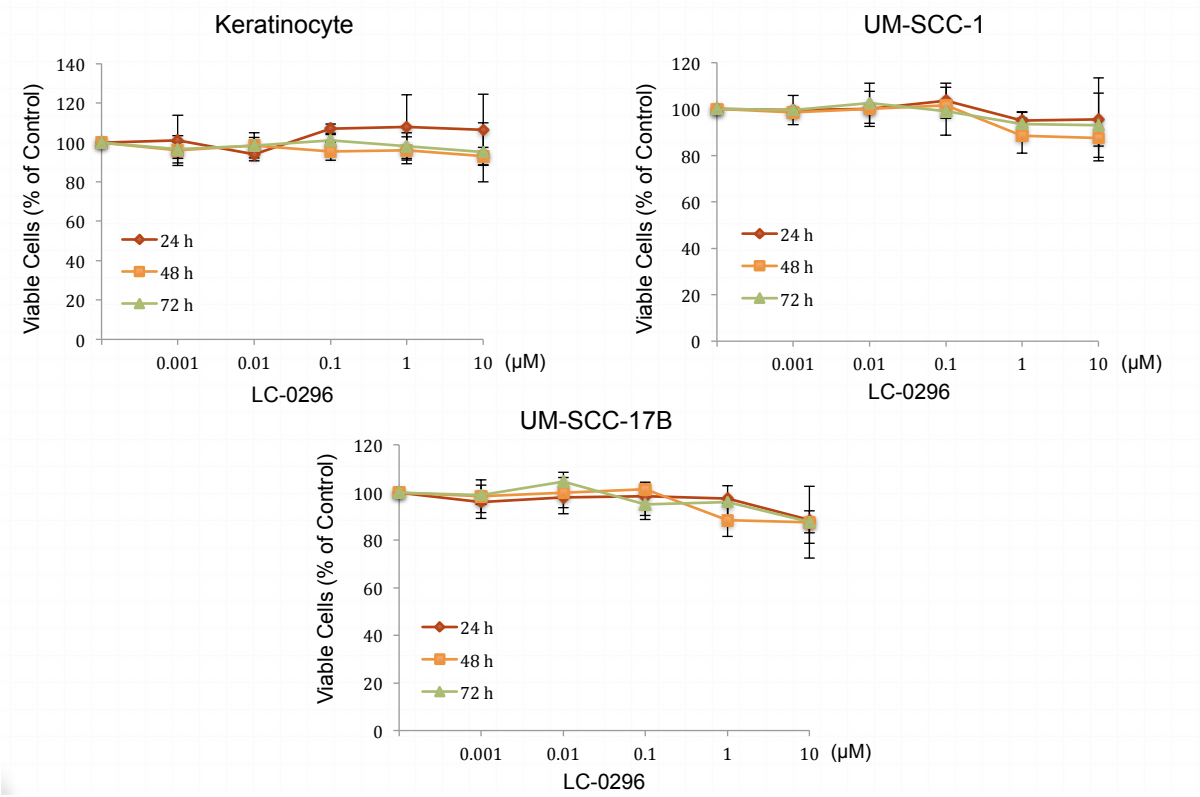


**Figure 4.S2: The effect of the SIRT3 inhibitor, LC-0296, on HNSCC cells and Keratinocytes using wide range of lower drug doses** (A) Normal human oral keratinocytes, (B) UM-SCC-1, and (C) UM-SCC-17B, were seeded in 96 well plate at 5 x$10^3$ cells/well, then treated with control (DMSO) or LC-0296, as indicated for 24, 48, and 72 h. Cell viability was then determined by the QUANT Cell Proliferation Assay Kit.

**Table 4.1: SIRT3 Enzymatic Activity *In vitro***

| SIRT3 Inhibitor | MW | SIRT1 $IC_{50}$ (μM) | SIRT2 $IC_{50}$ (μM) | SIRT3 $IC_{50}$ (μM) |
|---|---|---|---|---|
| LC-0296 | 542.58 | 67 | 33 | 3.6 |

**References**


1. http://www.cancer.org/ (Accessed 8-20-11).
2. http://www.cancer.ca/ (Accessed 8-22-11).
3. Saunders LR, Verdin E (2007) Sirtuins: critical regulators at the crossroads between cancer and aging. Oncogene 26: 5489-5504.
4. Michan S, Sinclair D (2007) Sirtuins in mammals: insights into their biological function. Biochem J 404: 1-13.
5. McGuinness D, McGuinness DH, McCaul JA, Shiels PG (2011) Sirtuins, bioageing, and cancer. Journal of aging research 2011: 235754.
6. Verdin E, Hirschey MD, Finley LW, Haigis MC (2010) Sirtuin regulation of mitochondria: energy production, apoptosis, and signaling. Trends Biochem Sci 35: 669-675.
7. Balcerczyk A, Pirola L (2010) Therapeutic potential of activators and inhibitors of sirtuins. BioFactors 36: 383-393.
8. Deng CX (2009) SIRT1, is it a tumor promoter or tumor suppressor? Int J Biol Sci 5: 147-152.
9. Lim CS (2006) SIRT1: tumor promoter or tumor suppressor? Med Hypotheses 67: 341-344.
10. Alhazzazi TY, Kamarajan P, Verdin E, Kapila YL (2011) SIRT3 and cancer: tumor promoter or suppressor? Biochim Biophys Acta 1816: 80-88.
11. Alhazzazi TY, Kamarajan P, Joo N, Huang JY, Verdin E, et al. (2011) Sirtuin-3 (SIRT3), a novel potential therapeutic target for oral cancer. Cancer 117: 1670-1678.
12. Fischel JL, Formento P, Milano G (2005) Epidermal growth factor receptor double targeting by a tyrosine kinase inhibitor (Iressa) and a monoclonal antibody (Cetuximab). Impact on cell growth and molecular factors. British journal of cancer 92: 1063-1068.
13. Chou TC, Talalay P (1984) Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Advances in enzyme regulation 22: 27-55.
14. Grenman R, Burk D, Virolainen E, Wagner JG, Lichter AS, et al. (1988) Radiosensitivity of head and neck cancer cells in vitro. A 96-well plate clonogenic cell assay for squamous cell carcinoma. Arch Otolaryngol Head Neck Surg 114: 427-431.
15. Carey TE, Van Dyke DL, Worsham MJ, Bradford CR, Babu VR, et al. (1989) Characterization of human laryngeal primary and metastatic squamous cell carcinoma cell lines UM-SCC-17A and UM-SCC-17B. Cancer Res 49: 6098-6107.
16. Szatrowski TP, Nathan CF (1991) Production of large amounts of hydrogen peroxide by human tumor cells. Cancer research 51: 794-798.
17. Toyokuni S, Okamoto K, Yodoi J, Hiai H (1995) Persistent oxidative stress in cancer. FEBS letters 358: 1-3.
18. Huang JY, Hirschey MD, Shimazu T, Ho L, Verdin E (2010) Mitochondrial sirtuins. Biochim Biophys Acta 1804: 1645-1651.
19. Lavu S, Boss O, Elliott PJ, Lambert PD (2008) Sirtuins--novel therapeutic targets to treat age-associated diseases. Nat Rev Drug Discov 7: 841-853.
20. Bosch-Presegue L, Vaquero A (2011) The dual role of sirtuins in cancer. Genes & cancer 2: 648-662.
21. Wu WS (2006) The signaling mechanism of ROS in tumor progression. Cancer metastasis reviews 25: 695-705.
22. Behrend L, Henderson G, Zwacka RM (2003) Reactive oxygen species in oncogenic transformation. Biochemical Society transactions 31: 1441-1444.
23. Pelicano H, Carney D, Huang P (2004) ROS stress in cancer cells and therapeutic implications. Drug resistance updates : reviews and commentaries in antimicrobial and anticancer chemotherapy 7: 97-110.
24. Pervaiz S, Clement MV (2004) Tumor intracellular redox status and drug resistance--serendipity or a causal relationship? Current pharmaceutical design 10: 1969-1977.
25. Qiu X, Brown K, Hirschey MD, Verdin E, Chen D (2010) Calorie Restriction Reduces Oxidative Stress by SIRT3-Mediated SOD2 Activation. Cell Metab 12: 662-667.
26. Tao R, Coleman MC, Pennington JD, Ozden O, Park SH, et al. (2010) Sirt3-mediated deacetylation of evolutionarily conserved lysine 122 regulates MnSOD activity in response to stress. Mol Cell 40: 893-904.

27. Kim HS, Patel K, Muldoon-Jacobs K, Bisht KS, Aykin-Burns N, et al. (2010) SIRT3 Is a Mitochondria-Localized Tumor Suppressor Required for Maintenance of Mitochondrial Integrity and Metabolism during Stress. Cancer Cell 17: 41-52.
28. Boonstra J, Post JA (2004) Molecular events associated with reactive oxygen species and cell cycle progression in mammalian cells. Gene 337: 1-13.
29. Arora-Kuruganti P, Lucchesi PA, Wurster RD (1999) Proliferation of cultured human astrocytoma cells in response to an oxidant and antioxidant. Journal of neuro-oncology 44: 213-221.
30. Chiao JW, Chung F, Krzeminski J, Amin S, Arshad R, et al. (2000) Modulation of growth of human prostate cancer cells by the N-acetylcysteine conjugate of phenethyl isothiocyanate. International journal of oncology 16: 1215-1219.
31. Albini A, D'Agostini F, Giunciuglio D, Paglieri I, Balansky R, et al. (1995) Inhibition of invasion, gelatinase activity, tumor take and metastasis of malignant cells by N-acetylcysteine. International journal of cancer Journal international du cancer 61: 121-129.
32. Trachootham D, Alexandre J, Huang P (2009) Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? Nature reviews Drug discovery 8: 579-591.
33. Montero AJ, Jassem J (2011) Cellular redox pathways as a therapeutic target in the treatment of cancer. Drugs 71: 1385-1396.

# CHAPTER V

## SIRT3 AND CANCER: TUMOR PROMOTER OR SUPPRESSOR?


## ABSTRACT

Sirtuins (SIRT1–7), the mammalian homologues of the Sir2 gene in yeast, have emerging roles in age-related diseases, such as cardiac hypertrophy, diabetes, obesity, and cancer. However, the role of several sirtuin family members, including SIRT1 and SIRT3, in cancer has been controversial. The aim of this review is to explore and discuss the seemingly dichotomous role of SIRT3 in cancer biology with particular emphasis on its potential role as a tumor promoter and tumor suppressor. This review will also discuss the potential role of SIRT3 as a novel therapeutic target to treat cancer.


### Introduction

Cancer is a leading cause of death worldwide, and the second cause of death in the United States after heart disease [1]. Despite advances in technology and improved therapeutic approaches to treat this devastating disease, shortcomings remain, especially in treating aggressive and metastatic disease and in predicting individual responses to treatment. These observations underscore the complexity of this disease and the need for personalized cancer therapy to increase the efficacy of treatment in individual cancer patients [2]. Therefore, discovering new pathways that regulate cancer processes is crucial to developing better approaches for cancer prevention and treatment.

The study of sirtuins (SIRT1–7) in cancer tumorigenesis and therapy is an exciting and promising new area in cancer research [3,4]. SIRT3 is of particular interest. It regulates both cell death and survival, and therefore, a controversy has emerged in the literature about its role as a tumor promoter and/or tumor suppressor. In this review, we will explore the controversy to provide the reader with a better

understanding of the factors that contribute to this issue, and to discuss future directions and the possibility of using SIRT3 as a novel therapeutic target to treat cancer.

**Sirtuins: an overview**

Sirtuins (SIRT1–7) are the mammalian homologues of the silent information regulator 2 (Sir2) first discovered in *Saccharomyces cerevisiae* as an $NAD^+$-dependent histone deacetylase (HDAC). They are classified as class III HDACs: they require $NAD^+$ as a cofactor to exert their biological function. They contain an evolutionarily conserved core domain, which is essential for their activity as NAD-dependent deacetylases or ADP-ribosyltransferases [5,6]. Sirtuin biology is complex, and sirtuins are widely expressed in normal tissues [7]. They are involved in a myriad of cellular and tissue functions, such as regulating oxidative stress, repairing DNA, increasing genomic stability, and affecting cell survival, apoptosis, development, metabolism, aging and longevity [3,4]. Some sirtuins are located in different cellular compartments (Fig. 5.1). Those in the same compartment, such as the mitochondrial SIRT3, 4, and 5, have different sequences and thus unique and diverse cellular functions and can interact with different targets [4,5,6].

SIRT1 is the best-characterized member of the mammalian sirtuins. It is located predominately in the nucleus and modulates cellular stress and survival by deacetylating p53 [8,9], FOXO, and Ku70 [10,11], thus promoting tumorigenesis. SIRT1 is thought to have a role in skin, colon, breast and lung cancer, via one or more of these mentioned targets [12,13,14,15,16]. It also regulates vascular endothelial homeostasis, thereby controlling angiogenesis and vascular function, [17]. Thus, it is likely crucial in regulating cell survival, and its functions may contribute to cancer tumorigenesis.

On the other hand, SIRT1 might be a tumor suppressor [18,19,20]. For example, SIRT1 mutant mice possess an impaired DNA repair response, genomic instability, and increased incidence of tumorigenesis. Moreover, SIRT1 levels were lower in breast cancer and hepatic cell carcinoma than in normal controls

[19]. These studies highlight the discrepancy in the literature about the biological functions of SIRT1 and underscore the complexity of sirtuin biology (See review by Deng *et al.* [21]).

SIRT2 is found in the cytosol, where it colocalizes with microtubules and deacetylates α-tubulin [22]. It controls cell-cycle progression [23] and is downregulated in human gliomas, suggesting a tumor suppressor role in brain cancer [24].

The gene for the nuclear protein SIRT6 is located on chromosome 19p13.3; a region frequently affected by chromosomal alterations in acute leukemia [25]. In addition, SIRT6-deficient mice possess an aging-like phenotype and genomic instability [26,27].

SIRT7, which is localized in the nucleolus and functions as a positive regulator of RNA polymerase I-mediated transcription, is required for cell proliferation and survival [28]. It is located on chromosome 17q25.3; a region frequently associated with chromosomal alterations in leukemias and lymphomas [29]. SIRT7 is also upregulated in breast and thyroid cancers [30,31,32].

The remaining three sirtuins, SIRT3, SIRT4, and SIRT5, are mitochondrial sirtuins [7,33]. Although SIRT4 lacks deacetylation activity, it has weak ADP-ribosyltransferase activity [34,35] and plays an important role in insulin regulation [36]. SIRT4 knockout mice are viable, fertile, and display no phenotype abnormalities, compared to wild-type littermates, but show increased levels of insulin secretion [34]. In contrast to SIRT1 and SIRT3, SIRT4 activity is downregulated by calorie restriction (CR) [34]. SIRT5 has less deacetylase activity than SIRT1-3 [37] and remains the least-characterized sirtuin. SIRT5 is located on chromosome 6p23, an area linked to numerous abnormalities associated with malignant diseases, such as acute myeloid leukemia [38]. In contrast to SIRT4- and SIRT5-deficient mice, SIRT3-deficient mice show greater mitochondrial hyperacetylation than wild-type mice, suggesting that SIRT3 is a key mitochondrial deacetylase [39].

**SIRT3 subcellular localization**

Determining SIRT3's subcellular localization is important for finding its targets and substrates, explaining its cellular functions, and identifying important signaling cascades that may involve it. Human SIRT3 is expressed as a full-length 44-kD protein that is targeted to the mitochondria by its N-terminal localization sequence [40]. In the mitochondria, SIRT3 is cleaved via the mitochondrial matrix processing peptidase (MPP) to a short 28-kD protein, which is important for SIRT3 enzymatic activity [40,41]. Others reported that both forms for SIRT3 are enzymatically active [42]. Although most studies support a mitochondrial localization for SIRT3 [7,39,40,41,43,44,45,46,47], others suggest that SIRT3 might be present in the nucleus [42,47,48]. In addition, Sundaresan *et al.* reported that, although the long form of SIRT3 is found in the nucleus, cytoplasm, and mitochondria, the short form is extensively localized in the mitochondria, and during cellular stress, levels of both forms are increased in the nucleus and mitochondria of cardiomyocytes [49]. Despite this controversy, one can conclude that SIRT3 exerts a major role in the mitochondria and might also have a role in other cellular compartments [50].

**4. SIRT3 and cell survival**

Mitochondria contain large numbers of key molecules that regulate cell survival, death, and metabolic pathways and help to control the balance between health and disease [4,51,52]. For example, SIRT3 is critical for maintaining mitochondrial integrity and function. Along with SIRT4, and SIRT5, SIRT3 is a mitochondrial sirtuin [33], and $SIRT3^{-/-}$ mice manifest hyperacetylated mitochondrial proteins, impaired fatty-acid oxidation, and reduced levels of ATP [39,53,54]. In an early report supporting a prosurvival role for SIRT3 *in vivo,* rodents fasted for 48 h had increased levels of the $NAD^+$ biosynthetic enzyme Nampt in their mitochondria. The activity of Nampt, a stress and nutrient-responsive protein involved in maintaining cell viability, is regulated by SIRT3 [55]. In addition, under genotoxic stress, mitochondrial SIRT3 and SIRT4 were required to protect against genotoxic cell death in human embryonic kidney (HEK293) and fibrosarcoma cell lines [55].

With its central role in mitochondrial biology, SIRT3 contributes to cell survival by modulating oxidative stress pathways. Benigni *et al.* demonstrated that knockout of the angiotensin II type 1 receptor, a gene responsible for promoting high blood pressure and various pathological conditions, such as heart, kidney and brain diseases, promoted longevity in mice [56]. This receptor knockout was associated with increased numbers of mitochondria, attenuation of oxidative stress, and upregulation of Nampt and SIRT3 levels. SIRT3 protects cardiomyocytes and HeLa cells from genotoxic and oxidative stress-mediated cell death. By binding to and deacetylating Ku70, SIRT3 augments Ku70-Bax interactions, prevents Bax translocation to the mitochondria, and prevents apoptosis during stress-mediated conditions [49]. In addition, SIRT3 protects the heart from cardiac hypertrophy, at least in part, by attenuating reactive oxygen species (ROS) [57] and/or by regulating the mitochondrial permeability transition pore (mPTP) via deacetylating Cyclophilin-D [58]. SIRT3 utilizes exogenous $NAD^+$ to block cardiac hypertrophy by activating the LKB1-AMP kinase pathway [59] (See review by Pillai *et. al.* [60]). Under CR conditions, SIRT3 deacetylates and activates superoxide dismutase 2 (SOD2), thus protecting cells from ROS-mediated cell damage [61]. SIRT3 also mediates deacetylation of the evolutionarily conserved lysine 122 needed for the activity of manganese superoxide dismutase (MnSOD) in response to oxidative stress, thus protecting cells from stress-mediated damage [62]. Furthermore, in neurons, SIRT3 acts as prosurvival factor, thus protecting neurons from excitotoxic injury such as *N*-methyl-D-aspartate (NMDA)-induced neuronal death [63].

SIRT3 also exerts a prosurvival role in multiple cancer pathways. The tumor suppressor, p53 was recently identified as a new target for SIRT3 deacetylation in bladder cancer [64]. SIRT3 rescued p53-induced growth arrest in human bladder tumor–derived EJ-p53 cells, supporting a prosurvival role for SIRT3 [64]. Ashraf *et al.* reported that increased transcriptional levels of SIRT3 were associated with lymph node–positive breast cancer, and SIRT3 expression was significantly higher in these samples than normal breast biopsies [30]. We recently reported that SIRT3 levels were significantly higher in oral squamous cell carcinoma (OSCC) cell lines and human OSCC tissue microarray samples (TMAs) than in

normal controls [65]. Furthermore, SIRT3 downregulation inhibited cell growth and proliferation and increased the sensitivity of OSCC cells to radiation and chemotherapy treatments. To further demonstrate the role of SIRT3 in oral cancer carcinogenesis *in vivo*, we used a floor-of-mouth oral cancer murine model that mimics human OSCC [66,67] to study the effect of SIRT3 downregulation on OSCC tumor growth in immunodeficient mice. Downregulating SIRT3 reduced tumor burden *in vivo*, implicating a prosurvival role for SIRT3 in oral cancer [65].

Anoikis, apoptotic cell death triggered by loss of extracellular matrix (ECM) contacts, is dysregulated in many chronic debilitating and fatal diseases, including cancer, and resistance to anoikis contributes to the development and progression of cancer [68,69,70]. We recently reported that anoikis activates a CD95/Fas-mediated signaling pathway regulated by receptor interacting protein (RIP), a kinase that shuttles between CD95/Fas-mediated cell death and integrin/FAK-mediated survival pathways in oral squamous cell carcinoma (OSCC) cells [69]. Interestingly, we found that, as OSCC cells become resistant to anoikis, their SIRT3 expression increases and their RIP expression decreases. These cells exhibit a greater tumor burden *in vivo*. Additionally, SIRT3 is highly expressed in OSCC tissues and cells, where its expression pattern is opposite to that of RIP expression (Kapila lab; unpublished data). Thus, these observations suggest that SIRT3 may play a role in mediating anoikis resistance and tumorigenesis.

In summary, SIRT3 promotes survival and protects several cell types from cellular damage by maintaining mitochondrial integrity and functions and by enhancing their resistance to stress-mediated cell death. Similarly, SIRT3 overexpression in cancer cells promotes survival signals while suppressing apoptotic signals, thereby enhancing tumorigenesis (Fig. 5.2).

**SIRT3, apoptosis, and cell death**

In contrast, other reports support a proapoptotic role for SIRT3. SIRT3 induces growth arrest and apoptosis in several colorectal carcinoma and osteosarcoma cells and in non-cancer human cell lines, such as retinal epithelial and lung fibroblast cells [71]. This action is mediated, in part, by SIRT3 modulation

of the JNK2 signaling pathway in these cell lines [71]. Interestingly, the same group reported earlier that SIRT1 and JNK2 function as constitutive suppressors of apoptosis in colorectal carcinoma [72]. Thus, SIRT1 and SIRT3 have opposite roles in colorectal carcinoma. A similar observation was made in neurons [73], however, the role of SIRT3 in neurons is still controversial [63]. In leukemia cell lines, treatment with Kaempferol, a flavonoid that auto-oxidizes and generates ROS, induces apoptosis via increasing Bax and SIRT3 levels and activating caspase-3 cascades [74].

Recently, SIRT3 was reported to suppress tumors. Kim *et al.* implanted $SIRT3^{-/-}$ mouse embryonic fibroblasts (MEFs) expressing Myc/Ras into the hind limbs of nude mice. After 3 weeks, these mice developed tumors, but mice implanted with $SIRT3^{+/+}$ Myc/Ras, $SIRT3^{-/-}$ Myc, or $SIRT3^{-/-}$ Ras MEFs did not. Importantly, SIRT3 knockout MEFs did not undergo spontaneous immortalization or possess a tumorigenic phenotype, unless they became immortalized by the action of Myc or Ras. This transformation-permissive phenotype was mediated by increased levels of ROS, chromosomal instability, and altered intracellular metabolism. Some $SIRT3^{-/-}$ mice developed mammary tumors over the 24-month observation period. In addition, SIRT3 expression was found to be decreased in commercially obtained TMAs of human breast cancer samples and in other cancers (glioblastoma, prostate, head and neck, and others), based on a review of gene expression data from other sources [75]. These findings suggest that SIRT3 is a tumor suppressor. In agreement with this report, others found that, in breast cancer patients, SIRT3 levels were lower or undetectable in most of the samples than in normal individuals, and specifically breast and ovarian cancers were frequently associated with focal deletion of the SIRT3 gene. Additionally, tumors lacking SIRT3 (SIRT3-KO-MEFs transformed with Ras and E1a oncogenes) grew faster and were bigger than transformed SIRT3-WT tumors in a xenograft model. This downregulation was associated with upregulation of hypoxia inducible factor-1 α (HIF1-α) targeted genes [76]. Similarly, Bell *et al*. also demonstrated a tumor suppressor role for SIRT3 in human colon carcinoma and osteosarcoma cells, via the ability of SIRT3 to negatively regulate ROS and HIF1-α [77].

In HEK-293 cells, the transient receptor potential melastatin-related channel 2 (TRPM2), a nonselective cation channel, confers susceptibility to cell death in response to oxidative stress. This cell death was reduced by treating with the general sirtuin chemical inhibitor, NAM, and with selective downregulation of both SIRT3 and SIRT2 [78], thus supporting a proapoptotic role for these sirtuins in the context of TRPM2 and oxidative stress. SIRT3 also deacetylates cyclophilin D, a protein required for hexokinase II binding to voltage-dependent anion channels (VDACs) to maintain mitochondrial integrity. Thus, in some cancer cells, SIRT3 induces hexokinase II to dissociate from the mitochondria and activate apoptosis. However, in non-transformed cells, activation of SIRT3 may prevent necrotic cell death [79]. From these findings, SIRT3 seems to function as a proapoptotic signal in some cancer and non-cancer cell lines, and it may prevent a transformation-permissive phenotype in certain normal cells, thus guarding the cell as a tumor suppressor (Fig. 5.2).

**SIRT3, metabolism, and cancer**

Metabolism is important in cancer development and prevention [80]. Cancer cells are metabolically active and need ATP to maintain their growth, proliferation, and survival [81,82]. Thus, understanding how specific regulators of metabolism are altered in cancer will be very helpful for developing therapies. Furthermore, cancer cells shift their mode of ATP/energy production from oxidative phosphorylation to glycolysis. This is called the "Warburg effect" [83,84]. However, cancer cells can switch between glycolysis and fatty acid oxidation, depending on the environment and substrate availability. This suggests that targeting one metabolic pathway may not be sufficient as a treatment strategy, since this may lead to resistance and more aggressive cancer phenotypes [85,86]

Mitochondria are important determinants of energy regulation, metabolic homeostasis, and cellular lifespan [52,87,88], and in the mitochondria, SIRT3 regulates numerous metabolic processes, such as fatty-acid oxidation, oxidative phosphorylation, and the TCA cycle. These observations implicate SIRT3 as a key regulator of cancer processes. Several studies have highlighted the role of SIRT3 in metabolism

and homeostasis and revealed new targets and substrates for SIRT3-dependent deacetylation [33,89]. However, few linked SIRT3-regulated metabolism to cancer. Those that addressed SIRT3 as a tumor suppressor demonstrated that $SIRT3^{-/-}$ mice have depleted levels of ATP: about 50% less in the heart, liver and kidney, and increased ROS production than in wildtype mice [54,75]. Indeed, increased ROS levels promote mutagenesis and genomic instability [90], as was the presumed case in $SIRT3^{-/-}$ mice [75]. In addition, SIRT3 critically regulates the Warburg effect. Thus, SIRT3 mediates metabolic destabilization of HIF1-α, a factor that regulates the metabolic shift to glycolysis in cancer cells, and its upregulation is associated with tumorigenesis [76,77].

SIRT3 regulates mitochondrial energy homeostasis. More specifically, it maintains ATP basal levels by regulating mitochondrial electron transport by deacetylating the 39-kD protein NDUFA9 [54] and succinate dehydrogenase [91]. Moreover, ATP synthase (ATP5A) and the chaperone protein HSP70, which protect against oxidative stress, are targets for SIRT3 [92]. SIRT3 also regulates fatty-acid oxidation by deacetylating long-chain acyl coenzyme A dehydrogenase (LCAD), thereby augmenting its enzymatic activity. Mice lacking SIRT3 have hyperacetylated LCAD and fatty-acid oxidation disorders during fasting, including reduced ATP levels, hypoglycemia, and cold intolerance [53]. SIRT3 downregulation in the hepatocyte cell line, HepG2, results in dysfunction in the electron transfer chain, reduction of mitochondrial membrane potential, and increased levels of ROS [47]. The first discovered target of SIRT3-mediated deacetylation was acetyl-CoA synthetase 2 (AceCS2). SIRT3 deacetylates and activates AceCS2, an enzyme important in converting acetate to acetyl-CoA in the presence of ATP and CoA, an enzyme required for TCA (Krebs) cycle initiation [93,94,95]. Since SIRT3 also modulates several enzymes of the TCA cycle, including isocitrate dehydrogenase 2 (IDH2), this is yet another mechanism by which SIRT3 helps to modulate energy production from carbohydrates, fats, and proteins [96]. In addition, under CR conditions, SIRT3 seems to modulate IDH2 function and protect the cell from oxidative stress-induced cell death [97]. Interestingly, mutations of IDH2 are associated with some cancer

types, such as gliomas and acute myeloid leukemias; however, the use of IDH2 as a potential therapeutic target is still controversial [98,99,100].

Shi *et al.* demonstrated that SIRT3 regulates thermogenesis by modulating mitochondrial proteins, such as peroxisome proliferator-activated receptor gamma coactivator-1alpha (PGC-1α) and the uncoupling protein 1 (UCP1) [44]. On the other hand, SIRT3 is a target for PGC-1α, whereby ROS levels are regulated in the cell [101]. PGC-1α itself has important metabolic roles, including regulating adaptive thermogenesis, gluconeogenesis, mitochondrial biogenesis, and respiration, and it protects cells against ROS generation and damage. PGC-1α binds to the SIRT3 promoter, enhancing its expression and downstream signaling pathways, including the activation of anti-oxidants SOD2 and catalase [101].

Moreover, SIRT3 levels are lower in obese mice than in normal littermates, thus implicating a role for SIRT3 in controlling obesity [44]. Additionally, *ob/ob* mice treated with leptin, a key hormone in regulating fat metabolism and energy expenditure, showed increased levels of SIRT3, further supporting a role for SIRT3 in controlling obesity [102]. SIRT3 regulates ketone body production during fasting via deacetylating the mitochondrial protein 3-hydroxy-3-methylglu-taryl CoA synthase 2 (HMGCS2) [103], providing further evidence of the important roles of SIRT3 in regulating cellular metabolism.

In aggregate, although few studies have linked SIRT3-mediated metabolism directly to cancer, all these studies support a role for SIRT3 as a critical regulator of metabolism in the mitochondria, which in turn, participates in cancer development or prevention. Additionally, age-related diseases share common risk factors and perhaps even redundancy in their mechanisms of pathology [104]. Therefore, determining the role of SIRT3 in those diseases may help better define its potential role in their etiology and in the development of novel therapeutics.

**Is SIRT3 a tumor promoter or suppressor?**

Cancer cells possess six common traits including self-sufficiency in growth signals, insensitivity to antigrowth signals, evading apoptosis, sustained angiogenesis, limitless replicative potential, and tissue

invasion and metastasis [105]. In addition, emerging hallmarks and enabling characteristics in cancer cells include dysregulation of cellular energy and avoidance of immune distraction, and the consequences of genomic instability and tumor-promoting inflammation are factors that contribute to creating a tumorigenic microenvironment, thus further facilitating and supporting the unique features of cancer cells phenotype [82]. The fact that SIRT3 can regulate most of these cancer processes, implicates SIRT3 as a novel potential therapeutic target to treat cancer. However, the discrepancy in the literature regarding the role of SIRT3 in cancer complicates how best to develop SIRT3 as a potential target for cancer therapy. It may be most appropriate to consider the cell-dependent context, the background of each cell line tested, and the influence of different dysregulated pathways in these cell lines, as in the case for SIRT1 in colon cancer [14].

The finding that SIRT3 is overexpressed in metabolically active tissues, such as the heart, where SIRT3 protects against genomic and stress-mediated apoptosis, at least in part, via ROS reduction and increases in Ku70-Bax interactions [49,57], is one mechanism by which cancer cells that overexpress SIRT3 similarly resist cell death. This was indeed demonstrated by the same group in the HeLa cervical cancer cell line [49].

SIRT3 is overexpressed to a greater extent in several human oral cancer cells and tissues than in normal controls, and SIRT3 downregulation in these cells inhibited OSCC cell growth and proliferation and enhanced radio- and chemo-therapeutic drug cytotoxicity. These observations suggest that these cells rely on SIRT3 signaling for survival. In addition, SIRT3 downregulation in OSCC cells *in vivo* reduced tumor burden in mice [65], further underscoring the critical role of SIRT3 in promoting survival and tumorigenesis in OSCC. Moreover, under suspension conditions, oral cancer cells aggregate to become anoikis resistant, maintaining their survival signals to escape suspension-induced cell death. One of those survival signals seems to be mediated by increased SIRT3 expression levels (Kapila lab, unpublished data).

Nampt protection against cell death, which was dependent on SIRT3 expression in fibrosarcoma cells [55], and the ability of SIRT3 overexpression to rescue p53-induced cell growth arrest in human bladder cancer [64], also demonstrate a prosurvival role for SIRT3 in these tumors. Overexpression of SIRT3 in lymph node-positive breast cancer, implicates a role for SIRT3 in advanced stages of breast cancer [30]. Together, these findings support a prosurvival role for SIRT3 in cancer, and the concept that SIRT3 functions as a tumor promoter in these tumors.

In contrast to the findings of Ashraf *et al.* [30], others showed that SIRT3 levels were lower in human breast cancer samples than in normal controls, and SIRT3 levels were further downregulated in advanced stages of breast cancer, supporting an opposite role for SIRT3 in breast cancer [75,76]. Interestingly, Kim *et al.* showed that MEFs from SIRT3 knockout mice did not become immortalized spontaneously. Instead, they required at least one oncogenic hit with either Myc or Ras to become immortalized *in vitro* and both Myc and Ras to develop tumors *in vivo* [75]. This suggests that environmental or genetic factors control how SIRT3 functions in cells. In addition, this group found that seven out of twenty SIRT3 knockout mice developed mammary tumors over a 24-month period, while none of the SIRT3 wildtype mice developed tumors [75]. However, after abrogating the function of an important cellular regulator such as SIRT3, 24 months is a reasonable time for genomic alterations and mutations to accumulate, which are critical initiating factors in the multistep process of cancer development [106,107]. Additionally, since SIRT3 is a critical regulator of the Warburg effect in cancer cells, downregulation of SIRT3, such as in breast cancer, would be associated with increased levels of ROS and a shift in metabolism toward glycolysis via the upregulation of HIF1-α and its targeted genes [76]. Moreover, in human colon carcinoma and osteosarcoma cells, SIRT3 also suppresses, ROS, HIF1-α and its targeted genes. Thus, colon carcinoma cells with stable knockdown of SIRT3 demonstrated enhanced tumorigenesis in a xenograft model, and augmented HIF1-α protein stability and transcriptional activity compared to controls [77].

In contrast, tumors with high levels of SIRT3 as part of their genomic and signaling dysregulation could take advantage of this overexpression to sustain their survival signals by downregulating ROS levels and maintaining high amounts of ATP/energy sufficient for their cancer cell machinery. In this regard, reduced levels of ROS were associated with higher levels of antiapoptotic mitochondrial proteins, such as Bcl-2 and Bcl-xL in OSCC cells [108]. Interestingly, ROS differentially regulates apoptosis and malignant transformation in several cancer types by regulating Bcl-2 [109,110]. Thus, superoxide plays a proapoptotic role by downregulating and degrading Bcl-2 proteins by ubiquitination, whereas nitric oxide (NO)-mediated S-nitrosylation of Bcl-2, abrogates its ubiquitination and subsequent proteosomal degradation [109,110]. In addition, Bcl-2 family proteins have been linked to resistance to cancer therapy in B-cell lymphomas and oral cancer [111,112,113]. It would be interesting to study the role of SIRT3 in modulating Bcl-2 family proteins in these cancer cell types.

Interestingly, SIRT1 is overexpressed in drug-resistant cancer cells, including neuroblastoma, osteosarcoma, mammary, and ovarian carcinomas [114]. Similarly we found that two OSCC cell lines (UM-SCC-1 and UM-SCC-17B) that are highly resistant to radiation and cisplatin treatment [115,116] become sensitive to low doses of both treatments only when SIRT3 levels were downregulated. This suggests a role for SIRT3 in resistance-mediated mechanisms in oral cancer. This resistance could be mediated by regulating Bcl-2 family proteins or related signaling cascades via SIRT3-ROS modulation. These mechanisms are currently under investigation in our laboratory.

Additionally, depending on SIRT3 levels, current evidence suggests that SIRT3 controls mitochondrial ROS directly or indirectly in the cell [60,89], thereby dictating the fate of a given cell type. Therefore, a cell either undergoes damage and assumes an environmentally-permissive neoplastic phenotype [62,75,76,117] or a protective/stress-mediated resistance and undergoes cell survival [49,57,61,62,97].

Interestingly, contrasting findings highlight the dichotomy of SIRT3's role in cancer processes in different cancer cell types. We found in oral cancer and others found in fibrosarcoma, cervical cancer, and

bladder cancer that SIRT3 was required to protect these tumors from stress-mediated cell death by various stimuli [55,57,64,65]. Others showed that SIRT3 was required to suppress tumorigenesis, and to induce stress-mediated cell death in tumors, including colorectal carcinoma, osteosarcoma, leukemia, and breast cancer [71,74,75,76,77]. Furthermore, the recent findings that SIRT3 might deacetylate cyclophilin-D, resulting in either enhancing apoptosis in transformed cells such as HeLa cells [79] or promoting survival and protecting against age-related cardiac hypertrophy [58], clearly show that SIRT3 may function differently depending on cell type. Even in normal cells such as neurons, SIRT3's role also seems to be controversial [63,73].

Thus, SIRT3's function varies in different normal and tumor tissues and may be cell- and tumor-type specific. Its role must not be generalized, but should be examined in each cancer type separately to determine whether it functions as a tumor promoter or suppressor. More importantly, the genetic or epigenetic alterations that underlie cancer initiation and progression may differ from individual to individual even within the same cancer type, resulting in a dysregulation of different signaling cascades that may or may not depend on SIRT3. These issues complicate our ability to predict the importance of SIRT3 in a given carcinogenic event. Therefore, looking at the bigger picture by screening cancer patients to analyze their genomic, epigenomic, proteomic, and metabolomic profile will help discover the dysregulated pathways that lead to a given disease, and thereby help maximize and personalize the therapeutic approaches for each patient.

**Sirtuins as potential therapeutic targets for cancer**

Several studies have implicated sirtuins as novel therapeutic targets for many age-related diseases, including cancer, but how sirtuins are involved in cancer is still not clear and controversial. In this review, we want to highlight the discrepancies in the literature about the roles of sirtuins in cancer, especially those of SIRT1 and SIRT3. A clear understanding of how individual sirtuins are involved in different

cancer types is important for assessing their potential in possible therapies. Sirtuins seem to be involved in tumorigenesis, and thus, sirtuin inhibitors/modifiers might have therapeutic benefit. Several inhibitors and activators of sirtuins have been tested in different cancer cell lines, but few have been tested *in vivo* [51]. The sirtuin inhibitors, sirtinol and splitomicin, induced senescence-like growth arrest in breast and lung cancers [118]. NAM, another sirtuin inhibitor, induced apoptosis in lung cancer [8]. We demonstrated that sirtinol and NAM inhibited cell growth and proliferation and induced apoptosis in oral cancer cells [65]. Treatment of B-cell lymphoma cells with cambinol, a SIRT1 inhibitor, inhibited tumor cell growth and induced apoptosis *in vitro,* and reduced tumor size compared to controls *in vivo* [119]. Moreover, cambinol sensitized lung cancer cells to the DNA-damaging agent etoposide, thus inducing cell death and etoposide-induced cell-cycle arrest [119] (See review by Balcerczyk *et al*. [120]).

Resveratrol, a polyphenol phytoalexin and natural component found in the skin of red grapes and red wine, works as an activator of sirtuins and possesses diverse natural therapeutic benefits, including cardiac protection, anti-inflammatory and anti-carcinogenic effects, preventing obesity, and promoting longevity [121,122]. These therapeutic benefits seem to work, at least in part, by activating SIRT1 and SIRT3, although it is not yet clear whether these effects are mediated by direct or indirect mechanisms [121,122,123,124]. Interestingly, resveratrol modulates both survival and death signals, depending on the administered dose *in vivo* [125]. At low doses (2.5 or 5 mg/kg for 14 days in rats), resveratrol provided cardiac protection and lower levels of apoptosis than controls. In contrast, at high doses (25 or 50 mg/kg), resveratrol hindered cardiac function and promoted apoptosis of cadiomyocytes. The former effect was mediated by augmenting survival signaling pathways, including p-Akt, NFκB, and Bcl-2 activation. The latter was mediated by switching on the death program by repressing the same pathways [125]. Moreover, resveratrol is the most studied sirtuin activator in cancer prevention. Many studies have shown resveratrol to be a natural anticarcinogenic agent, modulating different stages of cancer, including initiation, promotion, and progression in neuroblastoma, hepatoma, breast, lung, pancreatic, and prostate cancers *in vitro* or *in vivo* [126,127]. However, many of these studies yielded contradictory results even in the same

tumor type. We reasoned that these discrepancies could be due to the different experimental approaches used to examine resveratrol. For instance, some data were collected from mice and others from rats. Some studies used animal carcinogenesis models with different genetic backgrounds, others used different doses of resveratrol, and yet others used different time frames for drug administration. Resolving these discrepancies would be very helpful. In addition, these drugs are all generalized inhibitors or activators of several sirtuin family members. Thus, some redundancy or even opposing actions of some sirtuin functions may be expected. Furthermore, different tumors have different genetic backgrounds that differ from one person to another. This diversity may explain why one patient responds well to a specific treatment but another patient with the same type of cancer does not.

The rapidly evolving era of personalized medicine holds great promise for the future of cancer therapy. For example, the use of RNA interference (RNAi) as a specifically targeted therapeutic approach may be useful especially in combination with conventional treatments. Currently, studies using RNAi are still in early stages of clinical trials. RNAi has been tested in different types of cancer, such as lung, advanced liver, and chronic myeloid leukemia. We recently used an OSCC floor-of-mouth model in which mice treated with shRNA-modified-OSCC cells to reduce SIRT3 levels had a lower tumor burden than controls [65]. This demonstrates the usefulness of targeted gene knockdown as a potential therapeutic approach (See review by Phalon *et al.* [128]).

Class-I and II HDAC inhibitors have been tested in phase-I and II clinical trials with or without conventional chemotherapeutic drugs. The agents were well tolerated with low toxicity and yielded promising results. Some of the agents used include phenyl acetate, suberoylanilide hydroxamic acid, and Trichostatin to treat patients with solid tumors, hematologic malignancies, and advanced leukemias [129]. To our knowledge, there are no published reports on clinical trials using class-III HDAC inhibitors of sirtuins to treat cancer. However, class-III HDAC activators of sirtuins such as resveratrol are currently in early stages of clinical trials, and have been tested for safety and potential treatment of age-related

diseases such diabetes, neurodegenerative disorders and cancer (See references [130,131] about ongoing and published clinical trials).

**Conclusions**

We are just beginning to appreciate the role of sirtuins in treating age-related diseases, such as cancer. However, the current controversy regarding the role of SIRT3 in cancer, emphasizes the importance of examining this area further. Data support that SIRT3 can function as a tumor promoter or suppressor, depending on the cell- and tumor-type and the presence of different stress or cell death stimuli. Also, genetic and environmental factors underlying cancer initiation and development in each patient may contribute to this discrepancy. Thus, it is important to carefully examine the role of SIRT3 in each tumor type separately and in both *in vitro* and *in vivo* settings. Understanding the mechanistic differences in tumor types will enhance our knowledge of the complex biology of sirtuins in cancer and, ultimately, help us develop better therapeutic approaches. Therefore, screening cancer patients for genomic, proteomic, and metabolomic abnormalities and dysregulated signaling cascades to highlight important alterations might help identify useful targets for personalized cancer therapy and more successful cancer treatment.

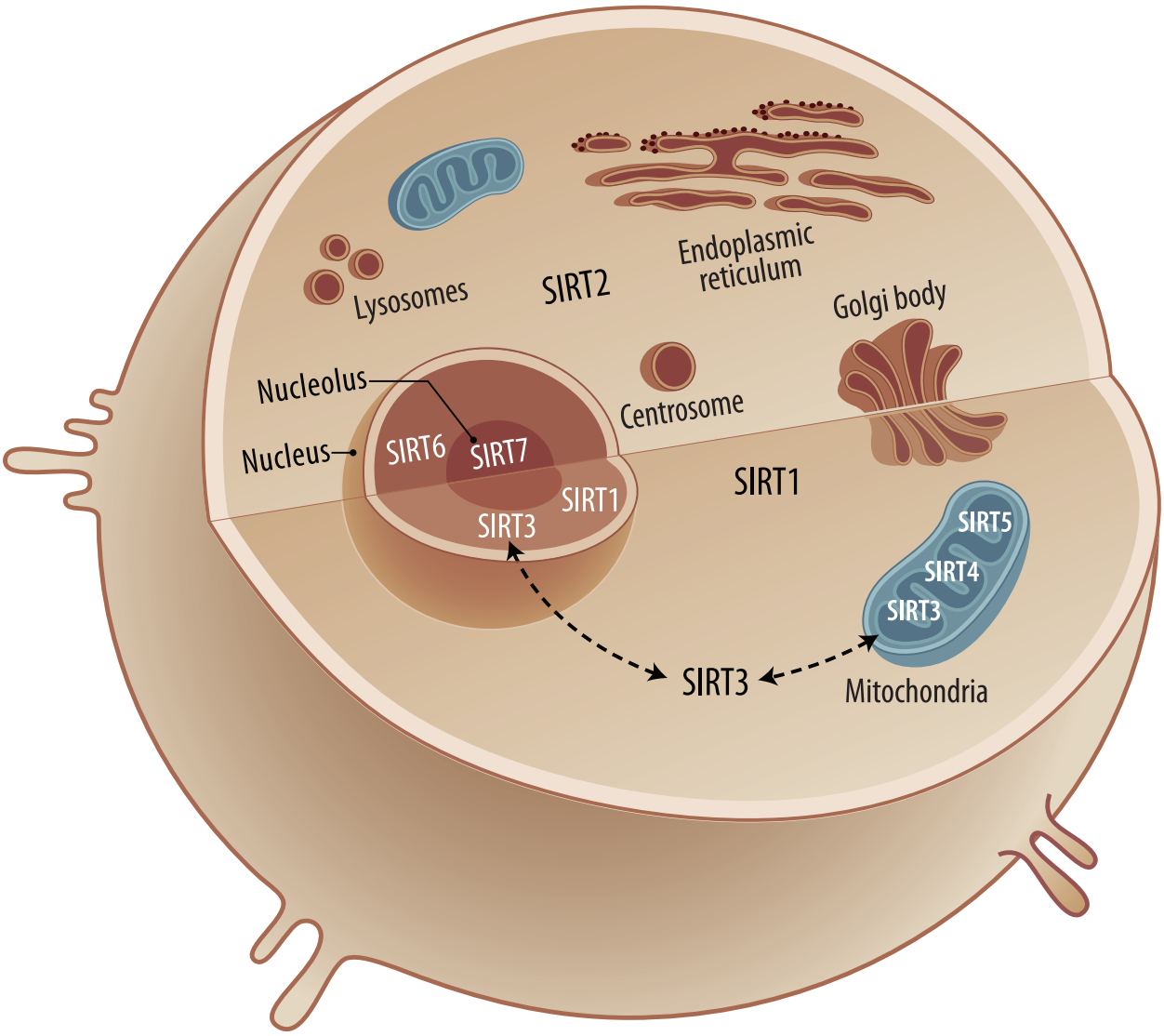


**Figure 5.1: Sirtuins subcellular localization:** SIRT1 is predominantly located in the nucleus, and also in the cytosol. SIRT2 is localized in the cytosol. SIRT3, SIRT4, and SIRT5 are mitochondrial proteins, but SIRT3 may also be found in the nucleus and cytosol under different cellular events. SIRT6 and SIRT7 are localized in the nucleus and nucleolus, respectively.

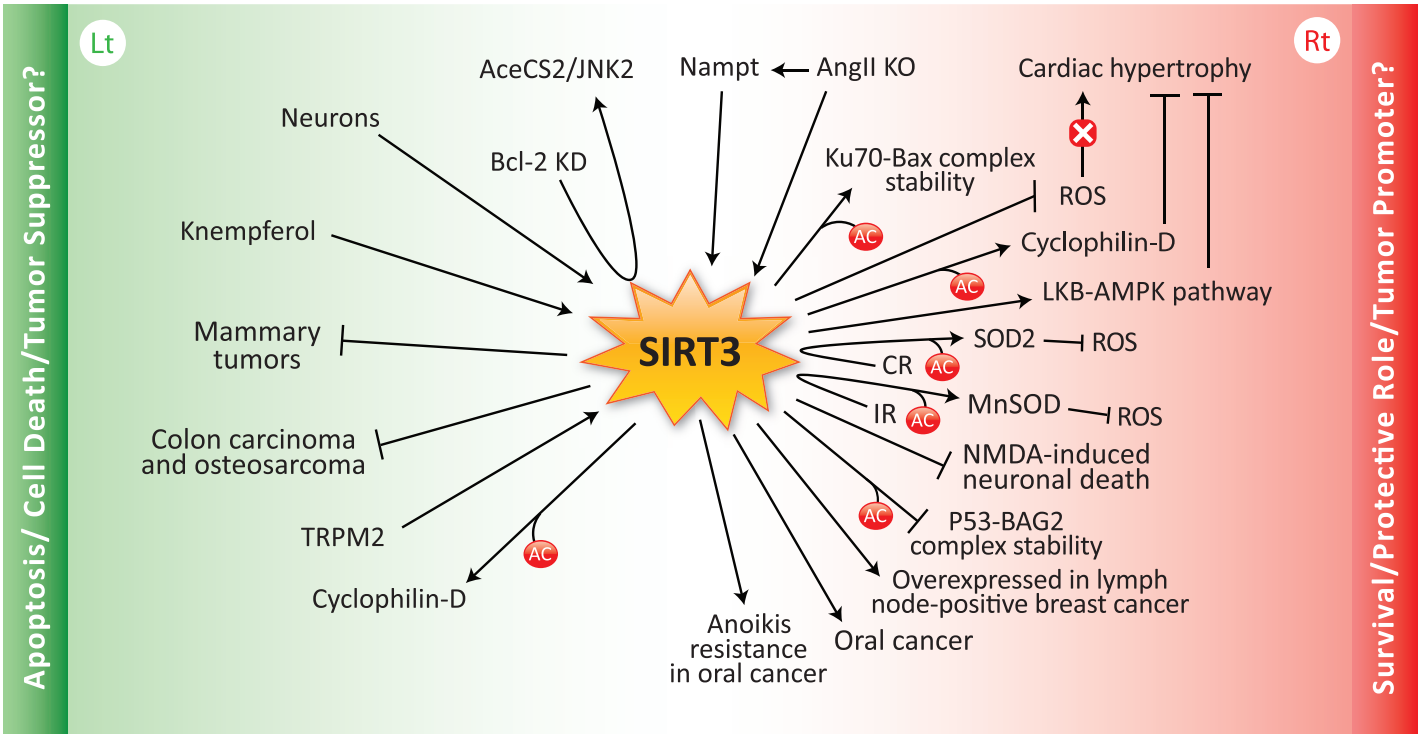


**Figure 5.2: SIRT3 diverse cellular functions.** ***(Rt)*** **The role of SIRT3 in survival, cell protection, and tumor promotion.** Nicotinamide phosphoribosyltransferase (Nampt), a stress and nutrient-responsive gene, protects against genotoxic cell death via SIRT3 upregulation. Gene knockout (KO) of Ang II promotes longevity in mice either directly or indirectly through Nampt or SIRT3 upregulation. SIRT3 deacetylates Ku70, augmenting Ku70-Bax interaction, thus attenuating apoptosis and promoting cell survival in cardiomyocytes. In the heart, SIRT3 also prevents cardiac hypertrophy by attenuating reactive oxygen species (ROS), deacetylating cyclophilin-D, and activating the anti-hypertrophic LKB1-AMP kinase signaling pathway. Calorie restriction (CR) prevents aging and age-related diseases by augmenting SIRT3 levels and functions in the cell, at least in part, by deacetylating and activating superoxide dismutase 2 (SOD2), thus protecting the cell from ROS-induced cell death. In addition, ionizing radiation (IR) in normal cells may enhance SIRT3-deacetylated-MnSOD activation, therefore, again protecting the cell from ROS-induced cell death. Although it is still controversial, in neurons SIRT3 seems to protect cells from excitotoxic injury such as *N*-methyl-D-aspartate (NMDA)-induced neuronal death. SIRT3 deacetylates p53, attenuating p53-BAG2 complex stability (BAG2; BCL2-associated athanogene 2), thus decreasing apoptosis. Lymph node–positive breast cancer is associated with increased levels of SIRT3. SIRT3 is also overexpressed in oral cancer and in anoikis-resistant oral squamous cell carcinoma (OSCC) cells, thus promoting OSCC cell survival and preventing anoikis-mediated cell death.. ***(Lt)*** **The role of SIRT3 in apoptosis, cell death, and tumor suppression.** In colorectal carcinoma, Bcl-2 knockdown (KD) was associated with SIRT3 upregulation and apoptosis by deacetylating AceCS2 and switching on the JNK2 signaling pathway. In neurons, low potassium (LK)-induced apoptosis is mediated by SIRT3. In leukemia, the treatment with Kaempferol, a flavonoid that auto-oxidizes and generates ROS, induces apoptosis by SIRT3 and Bax upregulation, thus switching on caspase-3 cascades and apoptosis. SIRT3 is downregulated in human breast cancer cells compared to normal controls, and $SIRT3^{-/-}$ mice developed mammary tumors over a 24-month period. In human colon carcinoma and osteosarcoma cells, SIRT3 also works as a tumor suppressor by suppressing ROS and HIF1-α. In HEK-293 cells, the transient receptor potential melastatin-related channel 2 (TRPM2), a nonselective cation channel induces cell death in response to oxidative stress via SIRT3.Unlike non-transformed cells, in some cancer cells, SIRT3 deacetylates cyclophilin-D, inducing the dissociation of hexokinase II/VDAC complex in the mitochondria, thus activating apoptosis.

**References**


[1] A.M. Minino, J. Xu, K.D. Kochanek, B. Tejada-Vera, Death in the United States, 2007, NCHS Data Brief (2009) 1-8.

[2] P. Workman, J. de Bono, Targeted therapeutics for cancer treatment: major progress towards personalised molecular medicine, Curr Opin Pharmacol 8 (2008) 359-362.

[3] L.R. Saunders, E. Verdin, Sirtuins: critical regulators at the crossroads between cancer and aging, Oncogene 26 (2007) 5489-5504.

[4] S. Michan, D. Sinclair, Sirtuins in mammals: insights into their biological function, Biochem J 404 (2007) 1-13.

[5] R.A. Frye, Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins, Biochem Biophys Res Commun 273 (2000) 793-798.

[6] M.C. Haigis, L.P. Guarente, Mammalian sirtuins--emerging roles in physiology, aging, and calorie restriction, Genes Dev 20 (2006) 2913-2921.

[7] E. Michishita, J.Y. Park, J.M. Burneskis, J.C. Barrett, I. Horikawa, Evolutionarily conserved and nonconserved cellular localizations and functions of human SIRT proteins, Mol Biol Cell 16 (2005) 4623-4635.

[8] J. Luo, A.Y. Nikolaev, S. Imai, D. Chen, F. Su, A. Shiloh, L. Guarente, W. Gu, Negative control of p53 by Sir2alpha promotes cell survival under stress, Cell 107 (2001) 137-148.

[9] H. Vaziri, S.K. Dessain, E. Ng Eaton, S.I. Imai, R.A. Frye, T.K. Pandita, L. Guarente, R.A. Weinberg, hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase, Cell 107 (2001) 149-159.

[10] A. Brunet, L.B. Sweeney, J.F. Sturgill, K.F. Chua, P.L. Greer, Y. Lin, H. Tran, S.E. Ross, R. Mostoslavsky, H.Y. Cohen, L.S. Hu, H.L. Cheng, M.P. Jedrychowski, S.P. Gygi, D.A. Sinclair, F.W. Alt, M.E. Greenberg, Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase, Science 303 (2004) 2011-2015.

[11] H.Y. Cohen, C. Miller, K.J. Bitterman, N.R. Wall, B. Hekking, B. Kessler, K.T. Howitz, M. Gorospe, R. de Cabo, D.A. Sinclair, Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase, Science 305 (2004) 390-392.

[12] D.M. Huffman, W.E. Grizzle, M.M. Bamman, J.S. Kim, I.A. Eltoum, A. Elgavish, T.R. Nagy, SIRT1 is significantly elevated in mouse and human prostate cancer, Cancer Res 67 (2007) 6612-6618.

[13] H. Ota, M. Akishita, M. Eto, K. Iijima, M. Kaneki, Y. Ouchi, Sirt1 modulates premature senescence-like phenotype in human endothelial cells, J Mol Cell Cardiol 43 (2007) 571-579.

[14] W. Stunkel, B.K. Peh, Y.C. Tan, V.M. Nayagam, X. Wang, M. Salto-Tellez, B. Ni, M. Entzeroth, J. Wood, Function of the SIRT1 protein deacetylase in cancer, Biotechnol J 2 (2007) 1360-1368.

[15] Y. Sun, D. Sun, F. Li, L. Tian, C. Li, L. Li, R. Lin, S. Wang, Downregulation of Sirt1 by antisense oligonucleotides induces apoptosis and enhances radiation sensitization in A549 lung cancer cells, Lung Cancer 58 (2007) 21-29.

[16] Y. Hida, Y. Kubo, K. Murao, S. Arase, Strong expression of a longevity-related protein, SIRT1, in Bowen's disease, Arch Dermatol Res 299 (2007) 103-106.

[17] M. Potente, S. Dimmeler, Emerging roles of SIRT1 in vascular endothelial homeostasis, Cell Cycle 7 (2008) 2117-2122.

[18] F. Gao, J. Cheng, T. Shi, E.T. Yeh, Neddylation of a breast cancer-associated protein recruits a class III histone deacetylase that represses NFkappaB-dependent transcription, Nat Cell Biol 8 (2006) 1171-1177.

[19] R.H. Wang, K. Sengupta, C. Li, H.S. Kim, L. Cao, C. Xiao, S. Kim, X. Xu, Y. Zheng, B. Chilton, R. Jia, Z.M. Zheng, E. Appella, X.W. Wang, T. Ried, C.X. Deng, Impaired DNA damage response, genome instability, and tumorigenesis in SIRT1 mutant mice, Cancer Cell 14 (2008) 312-323.

[20] M. Fu, M. Liu, A.A. Sauve, X. Jiao, X. Zhang, X. Wu, M.J. Powell, T. Yang, W. Gu, M.L. Avantaggiati, N. Pattabiraman, T.G. Pestell, F. Wang, A.A. Quong, C. Wang, R.G. Pestell, Hormonal control of androgen receptor function through SIRT1, Mol Cell Biol 26 (2006) 8122-8135.

[21] C.X. Deng, SIRT1, is it a tumor promoter or tumor suppressor?, Int J Biol Sci 5 (2009) 147-152.

[22] B.J. North, B.L. Marshall, M.T. Borra, J.M. Denu, E. Verdin, The human Sir2 ortholog, SIRT2, is an NAD+-dependent tubulin deacetylase, Mol Cell 11 (2003) 437-444.

[23] S.C. Dryden, F.A. Nahhas, J.E. Nowak, A.S. Goustin, M.A. Tainsky, Role for human SIRT2 NAD-dependent deacetylase activity in control of mitotic exit in the cell cycle, Mol Cell Biol 23 (2003) 3173-3185.

[24] M. Hiratsuka, T. Inoue, T. Toda, N. Kimura, Y. Shirayoshi, H. Kamitani, T. Watanabe, E. Ohama, C.G. Tahimic, A. Kurimasa, M. Oshimura, Proteomics-based identification of differentially expressed genes in human gliomas: down-regulation of SIRT2 gene, Biochem Biophys Res Commun 309 (2003) 558-566.
[25] U. Mahlknecht, A.D. Ho, S. Voelter-Mahlknecht, Chromosomal organization and fluorescence in situ hybridization of the human Sirtuin 6 gene, Int J Oncol 28 (2006) 447-456.
[26] R. Mostoslavsky, K.F. Chua, D.B. Lombard, W.W. Pang, M.R. Fischer, L. Gellon, P. Liu, G. Mostoslavsky, S. Franco, M.M. Murphy, K.D. Mills, P. Patel, J.T. Hsu, A.L. Hong, E. Ford, H.L. Cheng, C. Kennedy, N. Nunez, R. Bronson, D. Frendewey, W. Auerbach, D. Valenzuela, M. Karow, M.O. Hottiger, S. Hursting, J.C. Barrett, L. Guarente, R. Mulligan, B. Demple, G.D. Yancopoulos, F.W. Alt, Genomic instability and aging-like phenotype in the absence of mammalian SIRT6, Cell 124 (2006) 315-329.
[27] D.B. Lombard, B. Schwer, F.W. Alt, R. Mostoslavsky, SIRT6 in DNA repair, metabolism and ageing, J Intern Med 263 (2008) 128-141.
[28] E. Ford, R. Voit, G. Liszt, C. Magin, I. Grummt, L. Guarente, Mammalian Sir2 homolog SIRT7 is an activator of RNA polymerase I transcription, Genes Dev 20 (2006) 1075-1080.
[29] S. Voelter-Mahlknecht, S. Letzel, U. Mahlknecht, Fluorescence in situ hybridization and chromosomal organization of the human Sirtuin 7 gene, Int J Oncol 28 (2006) 899-908.
[30] N. Ashraf, S. Zino, A. Macintyre, D. Kingsmore, A.P. Payne, W.D. George, P.G. Shiels, Altered sirtuin expression is associated with node-positive breast cancer, Br J Cancer 95 (2006) 1056-1061.
[31] R. Frye, "SIRT8" expressed in thyroid cancer is actually SIRT7, Br J Cancer 87 (2002) 1479.
[32] F. de Nigris, J. Cerutti, C. Morelli, D. Califano, L. Chiariotti, G. Viglietto, G. Santelli, A. Fusco, Isolation of a SIR-like gene, SIR-T8, that is overexpressed in thyroid carcinoma cell lines and tissues, Br J Cancer 86 (2002) 917-923.
[33] J.Y. Huang, M.D. Hirschey, T. Shimazu, L. Ho, E. Verdin, Mitochondrial sirtuins, Biochim Biophys Acta 1804 (2010) 1645-1651.
[34] M.C. Haigis, R. Mostoslavsky, K.M. Haigis, K. Fahie, D.C. Christodoulou, A.J. Murphy, D.M. Valenzuela, G.D. Yancopoulos, M. Karow, G. Blander, C. Wolberger, T.A. Prolla, R. Weindruch, F.W. Alt, L. Guarente, SIRT4 inhibits glutamate dehydrogenase and opposes the effects of calorie restriction in pancreatic beta cells, Cell 126 (2006) 941-954.
[35] N. Ahuja, B. Schwer, S. Carobbio, D. Waltregny, B.J. North, V. Castronovo, P. Maechler, E. Verdin, Regulation of insulin secretion by SIRT4, a mitochondrial ADP-ribosyltransferase, J Biol Chem 282 (2007) 33583-33592.
[36] C. Argmann, J. Auwerx, Insulin secretion: SIRT4 gets in on the act, Cell 126 (2006) 837-839.
[37] E. Verdin, F. Dequiedt, W. Fischle, R. Frye, B. Marshall, B. North, Measurement of mammalian histone deacetylase activity, Methods Enzymol 377 (2004) 180-196.
[38] U. Mahlknecht, A.D. Ho, S. Letzel, S. Voelter-Mahlknecht, Assignment of the NAD-dependent deacetylase sirtuin 5 gene (SIRT5) to human chromosome band 6p23 by in situ hybridization, Cytogenet Genome Res 112 (2006) 208-212.
[39] D.B. Lombard, F.W. Alt, H.L. Cheng, J. Bunkenborg, R.S. Streeper, R. Mostoslavsky, J. Kim, G. Yancopoulos, D. Valenzuela, A. Murphy, Y. Yang, Y. Chen, M.D. Hirschey, R.T. Bronson, M. Haigis, L.P. Guarente, R.V. Farese, Jr., S. Weissman, E. Verdin, B. Schwer, Mammalian Sir2 homolog SIRT3 regulates global mitochondrial lysine acetylation, Mol Cell Biol 27 (2007) 8807-8814.
[40] B. Schwer, B.J. North, R.A. Frye, M. Ott, E. Verdin, The human silent information regulator (Sir)2 homologue hSIRT3 is a mitochondrial nicotinamide adenine dinucleotide-dependent deacetylase, J Cell Biol 158 (2002) 647-657.
[41] H.M. Cooper, J.Y. Huang, E. Verdin, J.N. Spelbrink, A new splice variant of the mouse SIRT3 gene encodes the mitochondrial precursor protein, PLoS ONE 4 (2009) e4986.
[42] M.B. Scher, A. Vaquero, D. Reinberg, SirT3 is a nuclear NAD+-dependent histone deacetylase that translocates to the mitochondria upon cellular stress, Genes Dev 21 (2007) 920-928.
[43] P. Onyango, I. Celic, J.M. McCaffery, J.D. Boeke, A.P. Feinberg, SIRT3, a human SIR2 homologue, is an NAD-dependent deacetylase localized to mitochondria, Proc Natl Acad Sci U S A 99 (2002) 13653-13658.
[44] T. Shi, F. Wang, E. Stieren, Q. Tong, SIRT3, a mitochondrial sirtuin deacetylase, regulates mitochondrial function and thermogenesis in brown adipocytes, J Biol Chem 280 (2005) 13560-13567.
[45] H.M. Cooper, J.N. Spelbrink, The human Sirt3 protein deacetylase is exclusively mitochondrial, Biochem J 411 (2008) 279-285.

[46] L. Jin, H. Galonek, K. Israelian, W. Choy, M. Morrison, Y. Xia, X. Wang, Y. Xu, Y. Yang, J.J. Smith, E. Hoffmann, D.P. Carney, R.B. Perni, M.R. Jirousek, J.E. Bemis, J.C. Milne, D.A. Sinclair, C.H. Westphal, Biochemical characterization, localization, and tissue distribution of the longer form of mouse SIRT3, Protein Sci 18 (2009) 514-525.
[47] J. Bao, Z. Lu, J.J. Joseph, D. Carabenciov, C.C. Dimond, L. Pang, L. Samsel, J.P. McCoy, Jr., J. Leclerc, P. Nguyen, D. Gius, M.N. Sack, Characterization of the murine SIRT3 mitochondrial localization sequence and comparison of mitochondrial enrichment and deacetylase activity of long and short SIRT3 isoforms, J Cell Biochem 110 (2010) 238-247.
[48] Y. Nakamura, M. Ogura, D. Tanaka, N. Inagaki, Localization of mouse mitochondrial SIRT proteins: shift of SIRT3 to nucleus by co-expression with SIRT5, Biochem Biophys Res Commun 366 (2008) 174-179.
[49] N.R. Sundaresan, S.A. Samant, V.B. Pillai, S.B. Rajamohan, M.P. Gupta, SIRT3 is a stress responsive deacetylase in cardiomyocytes that protects cells from stress-mediated cell death by deacetylation of Ku-70, Mol Cell Biol 28 (2008) 6384-6401.
[50] W.C. Hallows, B.N. Albaugh, J.M. Denu, Where in the cell is SIRT3?--functional localization of an NAD+-dependent protein deacetylase, Biochem J 411 (2008) e11-13.
[51] M. Porcu, A. Chiarugi, The emerging therapeutic potential of sirtuin-interacting drugs: from cell death to lifespan extension, Trends Pharmacol Sci 26 (2005) 94-103.
[52] I.R. Lanza, K.S. Nair, Mitochondrial function as a determinant of life span, Pflugers Arch 459 (2010) 277-289.
[53] M.D. Hirschey, T. Shimazu, E. Goetzman, E. Jing, B. Schwer, D.B. Lombard, C.A. Grueter, C. Harris, S. Biddinger, O.R. Ilkayeva, R.D. Stevens, Y. Li, A.K. Saha, N.B. Ruderman, J.R. Bain, C.B. Newgard, R.V. Farese, Jr., F.W. Alt, C.R. Kahn, E. Verdin, SIRT3 regulates mitochondrial fatty-acid oxidation by reversible enzyme deacetylation, Nature 464 (2010) 121-125.
[54] B.H. Ahn, H.S. Kim, S. Song, I.H. Lee, J. Liu, A. Vassilopoulos, C.X. Deng, T. Finkel, A role for the mitochondrial deacetylase Sirt3 in regulating energy homeostasis, Proc Natl Acad Sci U S A 105 (2008) 14447-14452.
[55] H. Yang, T. Yang, J.A. Baur, E. Perez, T. Matsui, J.J. Carmona, D.W. Lamming, N.C. Souza-Pinto, V.A. Bohr, A. Rosenzweig, R. de Cabo, A.A. Sauve, D.A. Sinclair, Nutrient-Sensitive Mitochondrial NAD(+) Levels Dictate Cell Survival, Cell 130 (2007) 1095-1107.
[56] A. Benigni, D. Corna, C. Zoja, A. Sonzogni, R. Latini, M. Salio, S. Conti, D. Rottoli, L. Longaretti, P. Cassis, M. Morigi, T.M. Coffman, G. Remuzzi, Disruption of the Ang II type 1 receptor promotes longevity in mice, J Clin Invest 119 (2009) 524-530.
[57] N.R. Sundaresan, M. Gupta, G. Kim, S.B. Rajamohan, A. Isbatan, M.P. Gupta, Sirt3 blocks the cardiac hypertrophic response by augmenting Foxo3a-dependent antioxidant defense mechanisms in mice, J Clin Invest 119 (2009) 2758-2771.
[58] A.V. Hafner, J. Dai, A.P. Gomes, C.Y. Xiao, C.M. Palmeira, A. Rosenzweig, D.A. Sinclair, Regulation of the mPTP by SIRT3-mediated deacetylation of CypD at lysine 166 suppresses age-related cardiac hypertrophy, Aging (Albany NY) 2 (2010) 914-923.
[59] V.B. Pillai, N.R. Sundaresan, G. Kim, M. Gupta, S.B. Rajamohan, J.B. Pillai, S. Samant, P.V. Ravindra, A. Isbatan, M.P. Gupta, Exogenous NAD blocks cardiac hypertrophic response via activation of the SIRT3-LKB1-AMP-activated kinase pathway, J Biol Chem 285 (2010) 3133-3144.
[60] V.B. Pillai, N.R. Sundaresan, V. Jeevanandam, M.P. Gupta, Mitochondrial SIRT3 and heart disease, Cardiovasc Res 88 (2010) 250-256.
[61] X. Qiu, K. Brown, M.D. Hirschey, E. Verdin, D. Chen, Calorie Restriction Reduces Oxidative Stress by SIRT3-Mediated SOD2 Activation, Cell Metab 12 (2010) 662-667.
[62] R. Tao, M.C. Coleman, J.D. Pennington, O. Ozden, S.H. Park, H. Jiang, H.S. Kim, C.R. Flynn, S. Hill, W. Hayes McDonald, A.K. Olivier, D.R. Spitz, D. Gius, Sirt3-mediated deacetylation of evolutionarily conserved lysine 122 regulates MnSOD activity in response to stress, Mol Cell 40 (2010) 893-904.
[63] S.H. Kim, H.F. Lu, C.C. Alano, Neuronal Sirt3 Protects against Excitotoxic Injury in Mouse Cortical Neuron Culture, PloS one 6 (2011) e14731.
[64] S. Li, M. Banck, S. Mujtaba, M.M. Zhou, M.M. Sugrue, M.J. Walsh, p53-Induced growth arrest is regulated by the mitochondrial SirT3 deacetylase, PLoS ONE 5 (2010) e10486.
[65] T.Y. Alhazzazi, P. Kamarajan, N. Joo, J.Y. Huang, E. Verdin, N.J. D'Silva, Y.L. Kapila, Sirtuin-3 (SIRT3), a novel potential therapeutic target for oral cancer, Cancer 117 (2011) 1670-1678.

[66] B. Henson, F. Li, D.D. Coatney, T.E. Carey, R.S. Mitra, K.L. Kirkwood, N.J. D'Silva, An orthotopic floor-of-mouth model for locoregional growth and spread of human squamous cell carcinoma, J Oral Pathol Med 36 (2007) 363-370.
[67] K.G. Wolter, S.J. Wang, B.S. Henson, S. Wang, K.A. Griffith, B. Kumar, J. Chen, T.E. Carey, C.R. Bradford, N.J. D'Silva, (-)-gossypol inhibits growth and promotes apoptosis of human head and neck squamous cell carcinoma in vivo, Neoplasia 8 (2006) 163-172.
[68] S. Sakamoto, N. Kyprianou, Targeting anoikis resistance in prostate cancer metastasis, Mol Aspects Med 31 (2010) 205-214.
[69] P. Kamarajan, J. Bunek, Y. Lin, G. Nunez, Y.L. Kapila, Receptor-interacting protein shuttles between cell death and survival signaling pathways, Mol Biol Cell 21 (2010) 481-488.
[70] J.M. Coates, J.M. Galante, R.J. Bold, Cancer therapy beyond apoptosis: autophagy and anoikis as mechanisms of cell death, J Surg Res 164 (2010) 301-308.
[71] S.J. Allison, J. Milner, SIRT3 is pro-apoptotic and participates in distinct basal apoptotic pathways, Cell Cycle 6 (2007) 2669-2677.
[72] J. Ford, M. Jiang, J. Milner, Cancer-specific functions of SIRT1 enable human epithelial cancer cell growth and survival, Cancer Res 65 (2005) 10457-10463.
[73] J.A. Pfister, C. Ma, B.E. Morrison, S.R. D'Mello, Opposing effects of sirtuins on neuronal survival: SIRT1-mediated neuroprotection is independent of its deacetylase activity, PLoS ONE 3 (2008) e4090.
[74] G. Marfe, M. Tafani, M. Indelicato, P. Sinibaldi-Salimei, V. Reali, B. Pucci, M. Fini, M.A. Russo, Kaempferol induces apoptosis in two different cell lines via Akt inactivation, Bax and SIRT3 activation, and mitochondrial dysfunction, J Cell Biochem 106 (2009) 643-650.
[75] H.S. Kim, K. Patel, K. Muldoon-Jacobs, K.S. Bisht, N. Aykin-Burns, J.D. Pennington, R. van der Meer, P. Nguyen, J. Savage, K.M. Owens, A. Vassilopoulos, O. Ozden, S.H. Park, K.K. Singh, S.A. Abdulkadir, D.R. Spitz, C.X. Deng, D. Gius, SIRT3 Is a Mitochondria-Localized Tumor Suppressor Required for Maintenance of Mitochondrial Integrity and Metabolism during Stress, Cancer Cell 17 (2010) 41-52.
[76] L.W. Finley, A. Carracedo, J. Lee, A. Souza, A. Egia, J. Zhang, J. Teruya-Feldstein, P.I. Moreira, S.M. Cardoso, C.B. Clish, P.P. Pandolfi, M.C. Haigis, SIRT3 Opposes Reprogramming of Cancer Cell Metabolism through HIF1alpha Destabilization, Cancer cell 19 (2011) 416-428.
[77] E.L. Bell, B.M. Emerling, S.J. Ricoult, L. Guarente, SirT3 suppresses hypoxia inducible factor 1alpha and tumor growth by inhibiting mitochondrial ROS production, Oncogene (2011).
[78] O. Grubisha, L.A. Rafty, C.L. Takanishi, X. Xu, L. Tong, A.L. Perraud, A.M. Scharenberg, J.M. Denu, Metabolite of SIR2 reaction modulates TRPM2 ion channel, J Biol Chem 281 (2006) 14057-14065.
[79] N. Shulga, R. Wilson-Smith, J.G. Pastorino, Sirtuin-3 deacetylation of cyclophilin D induces dissociation of hexokinase II from the mitochondria, J Cell Sci 123 (2010) 894-902.
[80] O. Resendis-Antonio, A. Checa, S. Encarnacion, Modeling core metabolism in cancer cells: surveying the topology underlying the warburg effect, PLoS One 5 (2010).
[81] B.M. Madhok, S. Yeluri, S.L. Perry, T.A. Hughes, D.G. Jayne, Targeting Glucose Metabolism: An Emerging Concept for Anticancer Therapy, Am J Clin Oncol (2010).
[82] D. Hanahan, R.A. Weinberg, Hallmarks of cancer: the next generation, Cell 144 (2011) 646-674.
[83] O. Warburg, F. Wind, E. Negelein, The Metabolism of Tumors in the Body, J Gen Physiol 8 (1927) 519-530.
[84] L.M. Ferreira, Cancer metabolism: The Warburg effect today, Exp Mol Pathol 89 (2010) 372-380.
[85] M. Buzzai, D.E. Bauer, R.G. Jones, R.J. Deberardinis, G. Hatzivassiliou, R.L. Elstrom, C.B. Thompson, The glucose dependence of Akt-transformed cells can be reversed by pharmacologic activation of fatty acid beta-oxidation, Oncogene 24 (2005) 4165-4173.
[86] Y. Liu, Fatty acid oxidation is a dominant bioenergetic pathway in prostate cancer, Prostate Cancer Prostatic Dis 9 (2006) 230-234.
[87] Y. Tsujimoto, Apoptosis and necrosis: intracellular ATP level as a determinant for cell death modes, Cell Death Differ 4 (1997) 429-434.
[88] H.D. Osiewacz, Role of mitochondria in aging and age-related disease, Exp Gerontol 45 (2010) 465.
[89] E. Verdin, M.D. Hirschey, L.W. Finley, M.C. Haigis, Sirtuin regulation of mitochondria: energy production, apoptosis, and signaling, Trends Biochem Sci 35 (2010) 669-675.
[90] B. Tudek, A. Winczura, J. Janik, A. Siomek, M. Foksinski, R. Olinski, Involvement of oxidatively damaged DNA and repair in cancer development and aging, Am J Transl Res 2 (2010) 254-284.

[91] H. Cimen, M.J. Han, Y. Yang, Q. Tong, H. Koc, E.C. Koc, Regulation of succinate dehydrogenase activity by SIRT3 in mammalian mitochondria, Biochemistry 49 (2010) 304-311.
[92] I.K. Law, L. Liu, A. Xu, K.S. Lam, P.M. Vanhoutte, C.M. Che, P.T. Leung, Y. Wang, Identification and characterization of proteins interacting with SIRT1 and SIRT3: implications in the anti-aging and metabolic effects of sirtuins, Proteomics 9 (2009) 2444-2456.
[93] B. Schwer, J. Bunkenborg, R.O. Verdin, J.S. Andersen, E. Verdin, Reversible lysine acetylation controls the activity of the mitochondrial enzyme acetyl-CoA synthetase 2, Proc Natl Acad Sci U S A 103 (2006) 10224-10229.
[94] W.C. Hallows, S. Lee, J.M. Denu, Sirtuins deacetylate and activate mammalian acetyl-CoA synthetases, Proc Natl Acad Sci U S A 103 (2006) 10230-10235.
[95] B.J. North, D.A. Sinclair, Sirtuins: a conserved key unlocking AceCS activity, Trends Biochem Sci 32 (2007) 1-4.
[96] C. Schlicker, M. Gertz, P. Papatheodorou, B. Kachholz, C.F. Becker, C. Steegborn, Substrates and regulation mechanisms for the human mitochondrial sirtuins Sirt3 and Sirt5, J Mol Biol 382 (2008) 790-801.
[97] S. Someya, W. Yu, W.C. Hallows, J. Xu, J.M. Vann, C. Leeuwenburgh, M. Tanokura, J.M. Denu, T.A. Prolla, Sirt3 Mediates Reduction of Oxidative Damage and Prevention of Age-Related Hearing Loss under Caloric Restriction, Cell 143 (2010) 802-812.
[98] K.E. Yen, M.A. Bittinger, S.M. Su, V.R. Fantin, Cancer-associated IDH mutations: biomarker and therapeutic opportunities, Oncogene 29 (2010) 6409-6417.
[99] B.C. Christensen, A.A. Smith, S. Zheng, D.C. Koestler, E.A. Houseman, C.J. Marsit, J.L. Wiemels, H.H. Nelson, M.R. Karagas, M.R. Wrensch, K.T. Kelsey, J.K. Wiencke, DNA methylation, isocitrate dehydrogenase mutation, and survival in glioma, Journal of the National Cancer Institute 103 (2011) 143-153.
[100] J. Zhu, J. Zuo, Q. Xu, X. Wang, Z. Wang, D. Zhou, Isocitrate dehydrogenase mutations may be a protective mechanism in glioma patients, Medical hypotheses 76 (2011) 602-603.
[101] X. Kong, R. Wang, Y. Xue, X. Liu, H. Zhang, Y. Chen, F. Fang, Y. Chang, Sirtuin 3, a new target of PGC-1alpha, plays an important role in the suppression of ROS and mitochondrial biogenesis, PLoS One 5 (2010) e11707.
[102] W. Zhang, M.A. Della-Fera, D.L. Hartzell, D. Hausman, C.A. Baile, Adipose tissue gene expression profiles in ob/ob mice treated with leptin, Life Sci 83 (2008) 35-42.
[103] T. Shimazu, M.D. Hirschey, L. Hua, K.E. Dittenhafer-Reed, B. Schwer, D.B. Lombard, Y. Li, J. Bunkenborg, F.W. Alt, J.M. Denu, M.P. Jacobson, E. Verdin, SIRT3 Deacetylates Mitochondrial 3-Hydroxy-3-Methylglutaryl CoA Synthase 2 and Regulates Ketone Body Production, Cell Metab 12 (2010) 654-661.
[104] N.M. Probst-Hensch, Chronic age-related diseases share risk factors: do they share pathophysiological mechanisms and why does that matter?, Swiss Med Wkly 140 (2010) w13072.
[105] D. Hanahan, R.A. Weinberg, The hallmarks of cancer, Cell 100 (2000) 57-70.
[106] E.R. Fearon, B. Vogelstein, A genetic model for colorectal tumorigenesis, Cell 61 (1990) 759-767.
[107] L. Foulds, The natural history of cancer, J Chronic Dis 8 (1958) 2-37.
[108] D. Li, E. Ueta, T. Kimura, T. Yamamoto, T. Osaki, Reactive oxygen species (ROS) control the expression of Bcl-2 family proteins by regulating their phosphorylation and ubiquitination, Cancer Sci 95 (2004) 644-650.
[109] N. Azad, A. Iyer, V. Vallyathan, L. Wang, V. Castranova, C. Stehlik, Y. Rojanasakul, Role of oxidative/nitrosative stress-mediated Bcl-2 regulation in apoptosis and malignant transformation, Ann N Y Acad Sci 1203 (2010) 1-6.
[110] N. Azad, A.K. Iyer, L. Wang, Y. Lu, D. Medan, V. Castranova, Y. Rojanasakul, Nitric oxide-mediated bcl-2 stabilization potentiates malignant transformation of human lung epithelial cells, Am J Respir Cell Mol Biol 42 (2010) 578-585.
[111] J.A. Bauer, B. Kumar, K.G. Cordell, M.E. Prince, H.H. Tran, G.T. Wolf, D.B. Chepeha, T.N. Teknos, S. Wang, A. Eisbruch, C.I. Tsien, S.G. Urba, F.P. Worden, J. Lee, K.A. Griffith, J.M. Taylor, N. D'Silva, S.J. Wang, K.G. Wolter, B. Henson, S.G. Fisher, T.E. Carey, C.R. Bradford, Targeting apoptosis to overcome cisplatin resistance: a translational study in head and neck cancer, Int J Radiat Oncol Biol Phys 69 (2007) S106-108.

[112] C. Stolz, G. Hess, P.S. Hahnel, F. Grabellus, S. Hoffarth, K.W. Schmid, M. Schuler, Targeting Bcl-2 family proteins modulates the sensitivity of B-cell lymphoma to rituximab-induced apoptosis, Blood 112 (2008) 3312-3321.
[113] H. Duan, C.A. Heckman, L.M. Boxer, Histone deacetylase inhibitors down-regulate bcl-2 expression and induce apoptosis in t(14;18) lymphomas, Mol Cell Biol 25 (2005) 1608-1619.
[114] F. Chu, P.M. Chou, X. Zheng, B.L. Mirkin, A. Rebbaa, Control of multidrug resistance gene mdr1 and cancer resistance to chemotherapy by the longevity gene sirt1, Cancer Res 65 (2005) 10183-10187.
[115] R. Grenman, D. Burk, E. Virolainen, J.G. Wagner, A.S. Lichter, T.E. Carey, Radiosensitivity of head and neck cancer cells in vitro. A 96-well plate clonogenic cell assay for squamous cell carcinoma, Arch Otolaryngol Head Neck Surg 114 (1988) 427-431.
[116] T.E. Carey, D.L. Van Dyke, M.J. Worsham, C.R. Bradford, V.R. Babu, D.R. Schwartz, S. Hsu, S.R. Baker, Characterization of human laryngeal primary and metastatic squamous cell carcinoma cell lines UM-SCC-17A and UM-SCC-17B, Cancer Res 49 (1989) 6098-6107.
[117] L.W. Oberley, Mechanism of the tumor suppressive effect of MnSOD overexpression, Biomed Pharmacother 59 (2005) 143-148.
[118] H. Ota, E. Tokunaga, K. Chang, M. Hikasa, K. Iijima, M. Eto, K. Kozaki, M. Akishita, Y. Ouchi, M. Kaneki, Sirt1 inhibitor, Sirtinol, induces senescence-like growth arrest with attenuated Ras-MAPK signaling in human cancer cells, Oncogene 25 (2006) 176-185.
[119] B. Heltweg, T. Gatbonton, A.D. Schuler, J. Posakony, H. Li, S. Goehle, R. Kollipara, R.A. Depinho, Y. Gu, J.A. Simon, A. Bedalov, Antitumor activity of a small-molecule inhibitor of human silent information regulator 2 enzymes, Cancer Res 66 (2006) 4368-4377.
[120] A. Balcerczyk, L. Pirola, Therapeutic potential of activators and inhibitors of sirtuins, BioFactors 36 (2010) 383-393.
[121] P. Signorelli, R. Ghidoni, Resveratrol as an anticancer nutrient: molecular basis, open questions and promises, J Nutr Biochem 16 (2005) 449-466.
[122] D.K. Das, S. Mukherjee, D. Ray, Resveratrol and red wine, healthy heart and longevity, Heart Fail Rev 15 (2010) 467-477.
[123] S. Rayalam, J.Y. Yang, S. Ambati, M.A. Della-Fera, C.A. Baile, Resveratrol induces apoptosis and inhibits adipogenesis in 3T3-L1 adipocytes, Phytother Res 22 (2008) 1367-1371.
[124] S. Mukherjee, D. Ray, I. Lekli, I. Bak, A. Tosaki, D.K. Das, Effects of Longevinex (modified resveratrol) on cardioprotection and its mechanisms of action, Can J Physiol Pharmacol 88 (2010) 1017-1025.
[125] J. Dudley, S. Das, S. Mukherjee, D.K. Das, Resveratrol, a unique phytoalexin present in red wine, delivers either survival signal or death signal to the ischemic myocardium depending on dose, J Nutr Biochem 20 (2009) 443-452.
[126] M. Athar, J.H. Back, X. Tang, K.H. Kim, L. Kopelovich, D.R. Bickers, A.L. Kim, Resveratrol: a review of preclinical studies for human cancer prevention, Toxicol Appl Pharmacol 224 (2007) 274-283.
[127] A. Bishayee, Cancer prevention and treatment with resveratrol: from rodent studies to clinical trials, Cancer prevention research 2 (2009) 409-418.
[128] C. Phalon, D.D. Rao, J. Nemunaitis, Potential use of RNA interference in cancer therapy, Expert Rev Mol Med 12 (2010) e26.
[129] M.R. Acharya, A. Sparreboom, J. Venitz, W.D. Figg, Rational development of histone deacetylase inhibitors as anticancer agents: a review, Mol Pharmacol 68 (2005) 917-932.
[130] K.R. Patel, E. Scott, V.A. Brown, A.J. Gescher, W.P. Steward, K. Brown, Clinical trials of resveratrol, Annals of the New York Academy of Sciences 1215 (2011) 161-169.
[131] http://www.clinicaltrials.gov/ct2/results?term=resveratrol, (Accessed April 25, 2011).

# CHAPTER VI

# CONCLUSIONS

## SUMMARY

The poor survival rate of head and neck cancer, which stands at approximately 50%, underscores the urgent need to explore new areas of research and develop new therapeutic drugs and approaches that can help improve the survival rate of head and neck cancer patients.

Sirtuins (SIRT1-7) have been extensively investigated for just over a decade. This field of study is an exciting area that seems to hold great promise toward enhancing our understanding of and aiding in the development of treatments for age-related diseases, such as diabetes, neurodegenerative disorders, heart disease, and cancer [1,2]. However, since sirtuin biology is still in an early stage of development, there are controversial viewpoints as to the significance of several sirtuins in the area of cancer biology. SIRT1 and SIRT3 seem to be at the focus of this controversy [3,4,5,6]. Thus, expanding the study of sirtuins in this area of research will advance the field and help us to better understand the mechanisms by which sirtuins can regulate different cancer processes. Sirtuins control cancer cell proliferation and survival, cell cycle progression, apoptosis, angiogenesis, and metabolism [2,7,8,9]. This implicates sirtuins as novel potential therapeutic targets to treat cancer [10]. Furthermore, the role of sirtuins in head and neck cancer has not yet been investigated. Thus, the aim of this dissertation work was to investigate whether sirtuins play a role in head and neck cancer tumorigenesis, and whether sirtuins can be used as novel sole or adjunctive therapeutic targets for HNSCC, thereby improving the survival rates for head and neck patients.

Here, we show for the first time a novel role for sirtuins, and specifically, sirtuin-3 (SIRT3), in head and neck cancer tumorigenesis. Our data support that of all the sirtuin family members, SIRT3 is specifically overexpressed in OSCC *in vitro* and *in vivo* compared to normal controls. SIRT3 downregulation enhanced the sensitivity of OSCC cells to both radiation and chemotherapeutic drugs. In addition, SIRT3 downregulation inhibited OSCC cell growth and proliferation *in vitro*, and reduced tumor burden *in vivo*.

We also show a link between SIRT3 and anoikis, apoptotic cell death triggered by loss of extracellular matrix (ECM) contacts. Interestingly, we found that SIRT3 and receptor interacting protein (RIP) are oppositely expressed in OSCC, and that OSCC cells escape anoikis by forming multicellular aggregates or oraspheres to maintain their survival compared to single cells, which undergo anoikis-mediated cell death. Additionally, anoikis-resistant OSCC cells that possess higher SIRT3 and lower RIP expression induced an increased tumor burden and incidence in mice unlike their adherent OSCC cell counterparts. Furthermore, stable suppression of SIRT3 inhibits anoikis-resistance and reduces tumor incidence. These findings suggest that SIRT3 may be a new potential therapeutic target to treat head and neck cancer patients.

To our knowledge, there are no published reports on clinical trials using class-III histone deacetylase inhibitors (HDAC) of sirtuins to treat cancer. Therefore, we next thought to investigate the use of a novel SIRT3 inhibitor, LC-0296, on HNSCC cells that are radio-resistant and originated from patients that resisted conventional therapy [11,12,13]. Interestingly, our data suggest that LC-0296 has a selective inhibitory effect on SIRT3 deacetylation activity compared to SIRT1 and SIRT2, the best characterized sirtuin family members with deacetylation activity [14]. Importantly, LC-0296, shows specificity toward retarding HNSCC cell proliferation and survival, and enhancing apoptosis, without affecting normal human oral keratinocytes. Additionally, LC-0296 not only worked as a single agent in HNSCC, but it can also be combined with and exert a synergistic effect with both radiation and cisplatin treatments, especially in cell

lines that were derived from patients that have shown resistance to conventional treatment. Because the literature supports that ROS levels are increased in cancer cells compared to normal cells [15,16], and SIRT3 plays a key role in regulating ROS levels in cells [9,17], we investigated whether LC-0296 would exert its inhibitory effect on SIRT3 deacetylation activity via modulating ROS levels in HNSCC cells. This, could them explain, in part, the selectivity of LC-0296's inhibitory effect on HNSCC cells compared to normal keratinocytes. In agreement with previously published reports in other cancer types [15,16], ROS levels were higher in HNSCC cells compared to normal oral keratinocytes. In addition, LC-0296 treatment further increases the levels of ROS in HNSCC cells compared to untreated controls. To further confirm that LC-0296 mediates its effect by modulating ROS levels in HNSCC cells, we next used N-Acetyl-Cysteine (NAC), a scavenger for ROS to challenge this mechanism. Indeed, NAC was able to inhibit the effects of LC-0296, in HNSCC cells, thus decreasing ROS levels. Importantly, the modulation of ROS levels in HNSCC cells by LC-0296, was associated with retarded cell survival and enhanced apoptosis. These data demonstrate that the SIRT3 inhibitor, LC-0296, mediates its inhibitory effect on HNSCC cells, at least in part, by modulating ROS levels. Our novel SIRT3 inhibitor, LC-0296, is the first generation of this type of drug. Our group is currently working on developing more potent and suitable versions of this drug that may be more applicable for testing within an *in vivo* setting and for potential future clinical trials.

Interestingly, inhibition of SIRT3 enzymatic activity had no additional effects on ROS levels in the context of stable SIRT3 suppression in HNSCC cells (Figure 6.1). This suggests that LC-0296 works mainly via inhibiting SIRT3 in HNSCC cells.

In agreement with other reports on the role of SIRT3 in fibrosarcoma, cervical cancer, and bladder cancer [18,19,20], our data demonstrate that SIRT3 plays a protective and prosurvival role in HNSCC via modulating cell survival and proliferation, and protecting HNSCC cells form apoptosis, at least in part, by modulating ROS levels.

Finally, in an effort further explore the role of SIRT3 in tumorigenesis, and to examine whether SIRT3 is a tumor promoter or suppressor, we reviewed these topics and presented them a review paper. This review highlights that SIRT3 might have a dual role in cancer, as a tumor promoter or suppressor, thus, it's function may vary in different normal and tumor tissues and may be cell- and tumor-type specific. Therefore, the role of SIRT3 as tumor promoter or suppressor should not be generalized, but should be examined in each cancer type separately to determine whether SIRT3 functions as a tumor promoter or suppressor. Additionally, a tumor's microenvironment, and its genomic dysregulation and signaling cascades, all differ from one tumor to another, and even from one patient to another within the same tumor type. Therefore, by looking at the bigger picture, screening cancer patients to analyze their genomic, epigenomic, proteomic, and metabolomic profile, seems to be necessary to help discover the dysregulated pathways that lead to a given disease, and thereby help maximize and personalize the therapeutic approaches for each patient.

In summary, this dissertation work highlights a novel role for sirtuins, and specifically, SIRT3 in head and neck cancer tumorigenesis. Our data suggest that the development of new therapy that specifically targets SIRT3, may be promising and helpful in the treatment of HNSCC, and may ultimately help improve the survival rate of head and neck cancer patients.

**FUTURE DIRECTIONS**

The goal of personalized cancer treatment is to achieve a more potent and targeted cancer therapy for each individual, which would be associated with fewer side effects, and would improve the survival rates of cancer patients. Thus, routinely screening cancer patients for genomic, proteomic, and metabolomic abnormalities and dysregulated signaling cascades to highlight important alterations, might help identify useful targets for personalized cancer therapy and more successful cancer treatment. Our data support an overexpression of SIRT3 in a majority of the oral cancer tissue. Therefore, in future directions our studies will be focusing on evaluating

large numbers of SIRT3 expression in tissue specimens obtained from head and neck cancer patients. We would like evaluate the databases associated with each tissue sample, to analyze patient demographics, including collect data that would include age, sex, medical history, alcohol and smoking status, HPV status, treatment used, and treatment outcomes. This would help to further determine if SIRT3 expression levels are strongly associate with HNSCC, and whether SIRT3 levels can be used as a biomarker/risk factor for diagnosing and evaluating treatment outcomes for patients with head and neck cancer.

In addition, our preliminary *in vitro* SIRT3 inhibitor studies support the development of new specific SIRT3 inhibitors that could aid in the treatment of patients suffering from head and neck cancer, and other cancer types that also possess high expression of SIRT3 as part of their genomic dysregulation. In this regard, we are still working on developing and testing a new generation of the SIRT3 inhibitor, LC-0296, that would be more potent and chemically stable for use within *in vivo* settings. We also generated a GPF-tagged stable cell line from the aggressive laryngeal metastatic UM-SCC-17B cell line, to be able to monitor the effect of the newly developed SIRT3 inhibitor on tumor cells *in vivo* (Figure 6.2).

The cellular localization of SIRT3 has also been controversial. Most reports support a mitochondrial localization [21,22,23,24,25,26,27], and some suggest a shuttling from the mitochondria to the nucleus or even a nuclear localization for SIRT3 that undergoes transportation to the mitochondria upon cellular stress [28,29,30]. Therefore, we sought to investigate the localization of SIRT3 in HNSCC cells in the presence or absence of cellular stress, as an initial step for future studies to help identify targets for SIRT3 deacetylation. Our preliminary data suggest that SIRT3 is a mitochondrial protein in HNSCC cells in the presence or absence of cisplatin treatment (Figure 6.3). In addition, our collaborators at UCSF, Eric Verdin and his colleagues, are experts in mitochondrial biology and acetylome analyses. Thus, we look forward to continuing this collaboration, to identify novel SIRT3 substrates in the context of

HNSCC to identify putative targets that might explain the prosurvival role of SIRT3 in head and neck cancer.

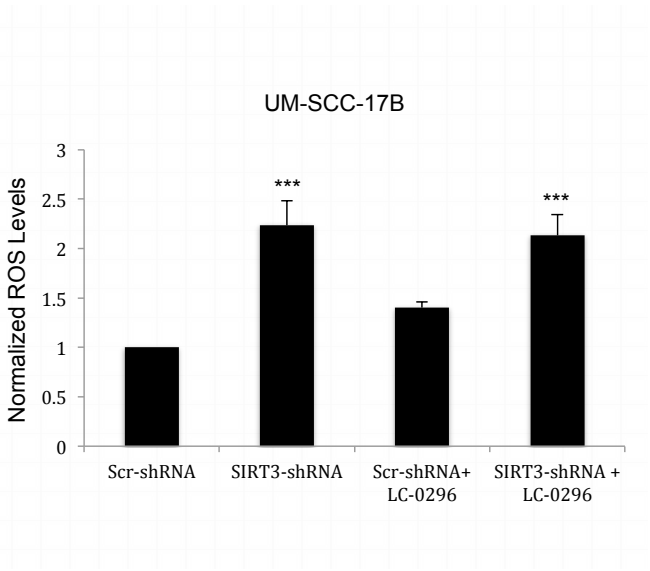


**Figure 6.1: Inhibition of SIRT3 enzymatic activity has no additional effects on ROS levels in the context of stable SIRT3 suppression in HNSCC cells.** ROS levels were measured in UM-SCC-17B cells that were stably transduced with scrambled-short hairpin RNA (Scr-shRNA) or SIRT3-shRNA after 10 days of selection using Puromycin, and after treatment with or without LC-0296 (50 μM) for 12 h.

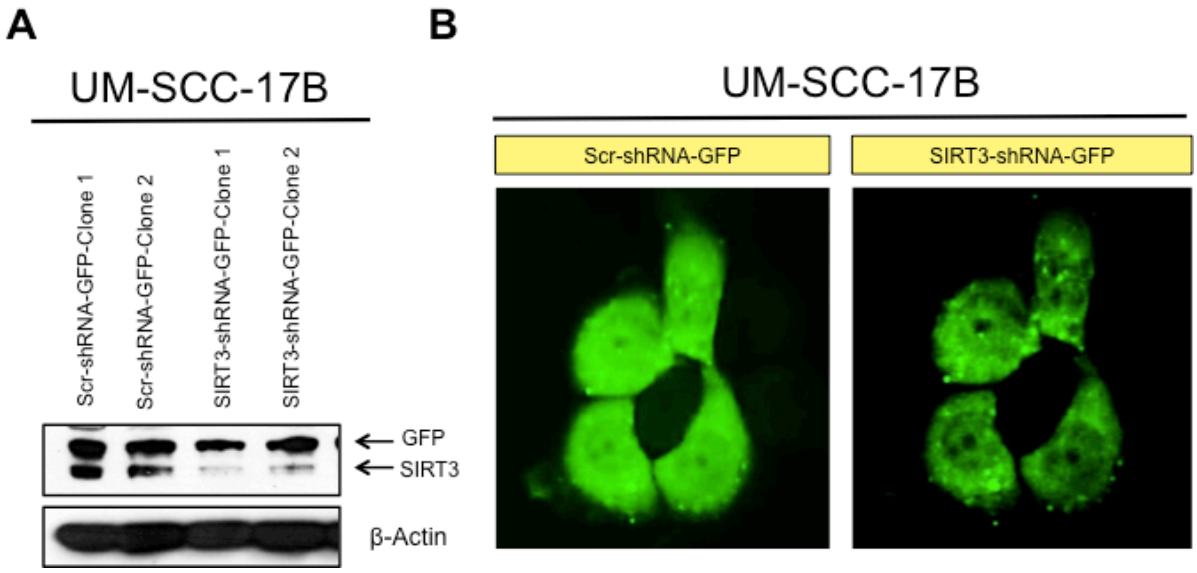


**Figure 6.2: The HNSCC cells (UM-SCC-17B) GFP-stable cell lines.** (A) Immunoblots showing SIRT3 expression and GFP levels in 2 clones from the UM-SCC-17B cell line that were stably transduced with scrambled-short hairpin RNA (Scr-shRNA-GFP) or SIRT3-shRNA-GFP after 10 days of selection using Puromycin. β-Actin served as loading control. (B) Fluorescent images showing stable transfection with the Scr-shRNA-GFP and SIRT3-shRNA-GFP in UM-SCC-17B cells.

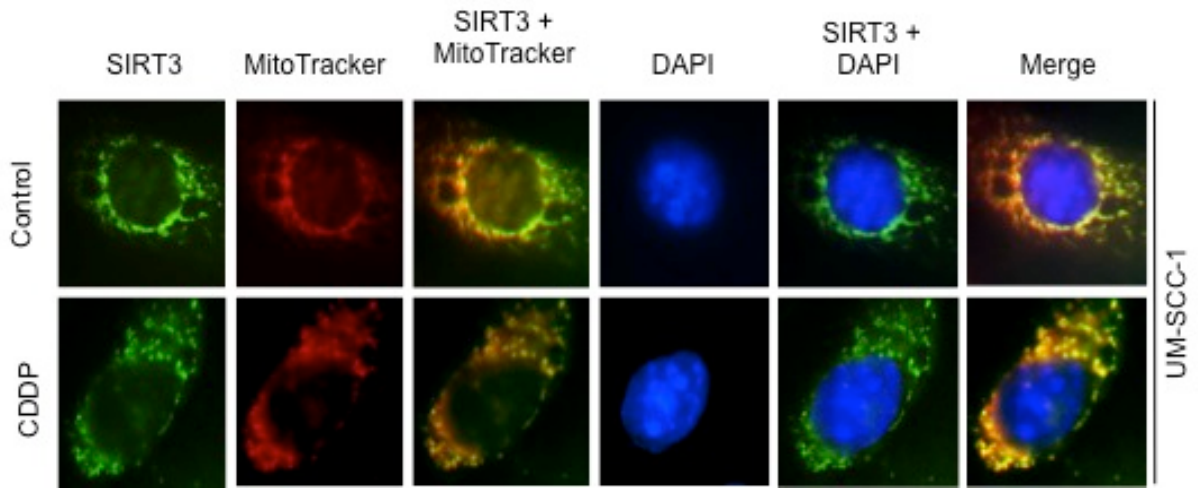


**Figure 6.3**: **SIRT3 cellular localization.** HNSCC (UM-SCC-1) cells were either treated with or without cisplatin (50 μM) for 8 h, stained with the mitochondrial marker, MitoTracker (red), and immunostained for SIRT3 (FITC, green), and the nucleus with DAPI (blue). Yellow represents colocalization of SIRT3 to the mitochondria (Original magnification x1000).

**References**


1. Lavu S, Boss O, Elliott PJ, Lambert PD (2008) Sirtuins--novel therapeutic targets to treat age-associated diseases. Nat Rev Drug Discov 7: 841-853.
2. Michan S, Sinclair D (2007) Sirtuins in mammals: insights into their biological function. Biochem J 404: 1-13.
3. Alhazzazi TY, Kamarajan P, Verdin E, Kapila YL (2011) SIRT3 and cancer: tumor promoter or suppressor? Biochim Biophys Acta 1816: 80-88.
4. Deng CX (2009) SIRT1, is it a tumor promoter or tumor suppressor? Int J Biol Sci 5: 147-152.
5. Lim CS (2006) SIRT1: tumor promoter or tumor suppressor? Med Hypotheses 67: 341-344.
6. Bosch-Presegue L, Vaquero A (2011) The dual role of sirtuins in cancer. Genes & cancer 2: 648-662.
7. Saunders LR, Verdin E (2007) Sirtuins: critical regulators at the crossroads between cancer and aging. Oncogene 26: 5489-5504.
8. McGuinness D, McGuinness DH, McCaul JA, Shiels PG (2011) Sirtuins, bioageing, and cancer. Journal of aging research 2011: 235754.
9. Verdin E, Hirschey MD, Finley LW, Haigis MC (2010) Sirtuin regulation of mitochondria: energy production, apoptosis, and signaling. Trends Biochem Sci 35: 669-675.
10. Balcerczyk A, Pirola L (2010) Therapeutic potential of activators and inhibitors of sirtuins. BioFactors 36: 383-393.
11. Grenman R, Burk D, Virolainen E, Wagner JG, Lichter AS, et al. (1988) Radiosensitivity of head and neck cancer cells in vitro. A 96-well plate clonogenic cell assay for squamous cell carcinoma. Arch Otolaryngol Head Neck Surg 114: 427-431.
12. Carey TE, Van Dyke DL, Worsham MJ, Bradford CR, Babu VR, et al. (1989) Characterization of human laryngeal primary and metastatic squamous cell carcinoma cell lines UM-SCC-17A and UM-SCC-17B. Cancer Res 49: 6098-6107.
13. Pekkola-Heino K, Kulmala J, Klemi P, Lakkala T, Aitasalo K, et al. (1991) Effects of radiation fractionation on four squamous cell carcinoma lines with dissimilar inherent radiation sensitivity. Journal of cancer research and clinical oncology 117: 597-602.
14. Verdin E, Dequiedt F, Fischle W, Frye R, Marshall B, et al. (2004) Measurement of mammalian histone deacetylase activity. Methods Enzymol 377: 180-196.
15. Szatrowski TP, Nathan CF (1991) Production of large amounts of hydrogen peroxide by human tumor cells. Cancer research 51: 794-798.
16. Toyokuni S, Okamoto K, Yodoi J, Hiai H (1995) Persistent oxidative stress in cancer. FEBS letters 358: 1-3.
17. Huang JY, Hirschey MD, Shimazu T, Ho L, Verdin E (2010) Mitochondrial sirtuins. Biochim Biophys Acta 1804: 1645-1651.
18. Yang H, Yang T, Baur JA, Perez E, Matsui T, et al. (2007) Nutrient-Sensitive Mitochondrial NAD(+) Levels Dictate Cell Survival. Cell 130: 1095-1107.
19. Sundaresan NR, Gupta M, Kim G, Rajamohan SB, Isbatan A, et al. (2009) Sirt3 blocks the cardiac hypertrophic response by augmenting Foxo3a-dependent antioxidant defense mechanisms in mice. J Clin Invest 119: 2758-2771.
20. Li S, Banck M, Mujtaba S, Zhou MM, Sugrue MM, et al. (2010) p53-Induced growth arrest is regulated by the mitochondrial SirT3 deacetylase. PLoS ONE 5: e10486.
21. Lombard DB, Alt FW, Cheng HL, Bunkenborg J, Streeper RS, et al. (2007) Mammalian Sir2 Homolog SIRT3 Regulates Global Mitochondrial Lysine Acetylation. Mol Cell Biol.
22. Cooper HM, Spelbrink JN (2008) The human SIRT3 protein deacetylase is exclusively mitochondrial. Biochem J 411: 279-285.
23. Schwer B, North BJ, Frye RA, Ott M, Verdin E (2002) The human silent information regulator (Sir)2 homologue hSIRT3 is a mitochondrial nicotinamide adenine dinucleotide-dependent deacetylase. J Cell Biol 158: 647-657.
24. Chen XJ, Clark-Walker GD (1994) sir2 mutants of Kluyveromyces lactis are hypersensitive to DNA-targeting drugs. Mol Cell Biol 14: 4501-4508.
25. Michishita E, Park JY, Burneskis JM, Barrett JC, Horikawa I (2005) Evolutionarily conserved and nonconserved cellular localizations and functions of human SIRT proteins. Mol Biol Cell 16: 4623-4635.

26. Shi T, Wang F, Stieren E, Tong Q (2005) SIRT3, a mitochondrial sirtuin deacetylase, regulates mitochondrial function and thermogenesis in brown adipocytes. J Biol Chem 280: 13560-13567.
27. Onyango P, Celic I, McCaffery JM, Boeke JD, Feinberg AP (2002) SIRT3, a human SIR2 homologue, is an NAD-dependent deacetylase localized to mitochondria. Proc Natl Acad Sci U S A 99: 13653-13658.
28. Scher MB, Vaquero A, Reinberg D (2007) SirT3 is a nuclear NAD+-dependent histone deacetylase that translocates to the mitochondria upon cellular stress. Genes Dev 21: 920-928.
29. Nakamura Y, Ogura M, Tanaka D, Inagaki N (2008) Localization of mouse mitochondrial SIRT proteins: shift of SIRT3 to nucleus by co-expression with SIRT5. Biochem Biophys Res Commun 366: 174-179.
30. Sundaresan NR, Samant SA, Pillai VB, Rajamohan SB, Gupta MP (2008) SIRT3 is a stress responsive deacetylase in cardiomyocytes that protects cells from stress-mediated cell death by deacetylation of Ku-70. Mol Cell Biol 28: 6384-6401.